Nitrogen Fixation: From Molecules to Crop Productivity

Proceedings of the 12th International Congress on Nitrogen Fixation, Foz do Iguaçu, Paraná, Brazil, September 12–17, 1999

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NITROGEN FIXATION: FROM MOLECULES TO CROP PRODUCTIVITY

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DEDICATION

The editors dedicate this volume to

Dr. Johanna Döbereiner
and
Dr. João Rui Jardim Freire

in acknowledgement of their contributions
to nitrogen-fixation research in Brazil.

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PREFACE

The 12th International Congress on Nitrogen Fixation was held in Foz do Iguaçu, Paraná State, Brazil, from the 12th to 17th of September, 1999. This volume constitutes the proceedings of this Congress and represents a compilation of the presentations by scientists from 38 countries who came to discuss the progress made, to exchange views and to collaborate. Since the first meeting in Pullman, Washington, USA, in 1974, this series of Congresses has been held three times in the United States, four times in Western Europe, in Australia, Mexico and Russia, and now for the first time in South America. Brazil was a most appropriate choice because Brazilian agriculture is especially dependent on biological nitrogen fixation. An important example is the soybean crop. The 30 million metric tons of grain produced each year relies solely on symbiotic nitrogen fixation. N-fertilizers are expensive in Brazil and, in the absence of the biological process, this crop would require 4-5 M tonnes of N at an annual cost of almost 1 billion dollars. Crop rotation, soil reclamation, forestry, forage mixed cropping, intercropping with grasses and legumes, associative nitrogen fixation with non-legume crops, such as sugarcane, and other agricultural practices in Brazil are also dependent on biological nitrogen fixation. The Brazilian experience with biological nitrogen fixation is a paradigm to be followed by all countries where nitrogen fertiliser is expensive or where present day environmental concerns demand a feasible, efficient and inexpensive alternative to chemical N-fertilizers.

In the biochemistry section, the analysis of the structure of the MoFe protein of *Klebsiella pneumoniae* at a resolution of 1.6 angstroms was described as was the complex between the altered *Azotobacter vinelandii* Fe protein Δ127L, which remains locked in the MgATP conformation in the absence of MgATP, and the MoFe protein. The intriguing possibility was raised that the Fe protein can be reduced by two electrons and transfer them both during a single encounter with the MoFe protein thereby improving energy expenditure with only two MgATPs hydrolysed per two electrons transferred. Other advances include the study of the FeMoco in non-protein surroundings, involving the binding of inhibitors and the reduction of substrates. These studies are paralleled by FTIR analysis of CO binding to the MoFe protein. The regulation of *nif* gene expression and nitrogen metabolism attracted a large number of contributions. Major advances in regulation have centered on the activation of the *nifA* gene and the activity of the NifA protein. Regulation of activity by NifL involves the GlnK protein in either a positive or negative manner, depending on the organism. The N-terminus of the *Herbaspirillum* protein inhibits the binding of the central plus C-terminal domain to the *nifH* promotor, whereas *nifL* probably prevents NifA interacting with the RNA polymerase. PII of *Herbaspirillum,* which activates NifA, has a structure similar to the GlnK of *Escherichia coli.* Autoactivation of the *nifA* gene in *Herbaspirillum* is inhibited by IHF, which acts positively and negatively, to regulate *nifA* expression. The role of the σ^N protein has been studied in depth.

A big shift has been made from genes to genomes. The sequence of the sym plasmid of NGR 234 has been completed as has the genome of *Rhodobacter capsulatus.* Other genomes under investigation are those of *Sinorhizobium meliloti, Bradyrhizobium japonicum,* the symbiosis island of a *Mesorhizobium loti* and the symbiotic plasmid of *Rhizobium etli.* Plant genomes that have been analyzed include *Medicago truncatula, Lotus japonicus* and soybean.

Interest persists in the associative and endophytic bacteria and evidence is growing that the endophytic bacteria, such as *Acetobacter diazotrophicus, Herbaspirillum* and *Azoarcus* species, colonize intercellular spaces in the cortex and also xylem vessels. The range of plant species hosting *Azospirillum, Herbaspirillum* and *Burkholderia,* as well as undefined species has increased. Clear evidence has been obtained that nitrogenase is expressed inside sugarcane plants harbouring *Acetobacter diazotrophicus* and inside rice harbouring *Azoarcus* spp. Because nitrogenase is expressed under these conditions, biological nitrogen fixation presumably contributes to bacterial growth and possibly to plant growth. However, the positive contribution of these organisms to plant growth goes beyond just nitrogen fixation.

Nod factors (LCO's) were the major focus concerning the control of nodulation in the legume symbiosis. Purified Nod factors, together with expression of the *enod40* gene, activate the inner cortical cells for the cell division necessary for nodule formation. Differentiation of meristematic cells involves arrest of cell division, an increase in cell size and infection by bacteroids. A plant cell regulator gene *ccs52* controls cell-division arrest and is essential for nodule formation. Lectin-like NTPases appear to be necessary for nodulation, possibly as Nod factor-binding proteins. A key issue in the session on metabolism of symbiosomes concerned alanine, rather than ammonium, as the major N exporter from the nodule to the plant. It is likely that either ammonium or alanine can be excreted, depending on the physiological state of the bacteroid. Interest continues to be high regarding both the nodule malic enzyme and the peribacteroid membrane.

New molecular tools are enabling progress in the study of molecular phylogeny and biological diversity. Several studies show similarities between N_2-fixing species and other species and genera not reported as N_2 fixers. Much effort is being expended in trying to unravel and comprehend the biodiversity of diazotrophic bacteria.

Agricultural practices emphasized the benefits of nitrogen fixation for improving soil fertility and increase yield, for playing an important role in the sustainable management of agricultural systems, and in soil reclamation. Phosphorus remains a key nutrient to improve the effectiveness of the nitrogen-fixation process and, therefore, the important role played by mycorrhizae was emphasized. Approaches using the inoculation of selected strains, improvement of inoculation technology, co-inoculation of microorganims and several other practices are being successfully used in several countries.

A final session was devoted to predictions of future research directions in nitrogen fixation for the 21st century. These included the possibility of establishing active nitrogen fixation in plants, the establishment of nitrogen-fixing symbioses with rice, and the use of transgenic legumes to combat environmental stress, including herbicide tolerance and resistance to pests and diseases.

We want to express our deep thanks to all those who worked hard and long to help us in the organization of this Congress. We want to emphasize our gratitude for the financial support that we received from all the institutions that believe in our work. Finally, we want to thank our families and friends for their moral support during the last two years.

Curitiba, September 1999

Fábio O. Pedrosa
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KEYNOTE LECTURE

NITROGEN FIXATION IN PERSPECTIVE

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1. Introduction

One of life's greatest paradoxes is that all living things depend on a source of utilizable nitrogen but only some of the smallest members of this community can produce it from the "sea" of otherwise inert, molecular nitrogen (N_2) that surrounds and saturates us. This "fixed" nitrogen utilized in all its forms comprises less than 0.0007% of the nitrogen on Earth and in its atmosphere. Nitrogen fixation is that part of the geobiochemical nitrogen cycle that drives the conversion of atmospheric N_2 to ammonia, whereas nitrification and denitrification act to return nitrogen to the atmosphere as N_2. N_2 is fixed by abiological, natural processes, including lightning, combustion and volcanism, which account for about 10% of the annual fixation. Man-made processes, mainly industrial ammonia production, contribute about 25% of the total annual fixation (Newton, 1996). Biological nitrogen fixation at about 65% of total annual fixation is, therefore, the main provider of fixed nitrogen and so is the major sustainer of life on Earth.

2. When and How did Biological Nitrogen Fixation Evolve?

Nitrogen fixation likely evolved when the geochemical reserves of fixed-N in the biosphere were depleted. So, when in geological time did these fixed-N reserves become limiting? When did the situation become ripe for diazotrophy? The simple answer is that no one knows. The considerable uncertainty about when prokaryotes acquired the ability to fix N_2 is highlighted by the opposing views of the composition of the pre-biological paleoatmosphere. It may have been strongly reducing and contained large amounts of ammonia, methane, carbon monoxide and hydrogen sulfide (Hart, 1979). Alternatively, it may have been more mildly reducing, with very little of these three gases, and contained N_2, carbon dioxide and water vapor (Levine, Augustsson, 1983). This lack of agreement makes it effectively impossible to estimate how long the fixed-N sources, which would be accessible after the biosphere arose, persisted. Even so, once free O_2 appeared, any geochemical ammonia would react to form N_2 and nitrogen oxides. Eventually, the availability of nitrogen oxides would limit the growth of organisms, leading to selective pressure and the appearance of diazotrophy. This scenario suggests that assimilatory nitrate and nitrite reduction pre-dated diazotrophy but it does not necessarily indicate that O_2-producing photosynthesis also pre-dated diazotrophy. Free O_2, at much lower than present-day levels, could have been produced abiologically by photolysis of water at a much earlier geological time (Towe, 1983). However, the appearance of non-dioxygenic phototrohy, driven by the sedimentation (and loss) of usable organic C at a rate faster than its non-biological synthesis, would have contributed to the pressure for diazotrophy (Sprent, Raven, 1985). The photosynthetically-driven carbon-sulfur/carbon-oxygen cycles would have out-paced the inputs from the abiological fixed-N sources. However, this suggestion does not mean that phototrophy was a requirement for the first N_2 fixers; they could easily have been content to assimilate the photosynthate of others. In any case, the high sensitivity of nitrogenase to denaturation by free O_2 suggests that it evolved on Earth under anoxic conditions where such sensitivity would not be a liability. Alternatively, of course, this high reactivity towards O_2 may be an unavoidable prerequisite for

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effective biological N_2 fixation. A completely different viewpoint (Wächtershäuser, 1988) suggests the existence of an aboriginal "nitrogenase", in the form of a pyrite-forming Fe-S cluster, which produced C-bound nitrogen rather than ammonia. This view of the origin of life has this “nitrogenase” fixing N_2 prior to the existence of enzymes and suggests that nitrogen fixation is very ancient indeed.

A logical extension of this last theory implies that early protein-like materials sequestered this entity and gave rise to the Fe-only nitrogenase (Chisnell et al., 1988), which would then be the forerunner of the three nitrogenases recognized today. Some support for this view comes from a consideration of the occurrence of Mo and W in enzymes in meso-, thermo- and hyperthermo-philes. If indeed, the early Earth were hot, hyperthermophiles would have appeared first, followed by thermophiles and, finally, by mesophiles as the Earth cooled. Of the usual Mo/W hydroxylases and oxidoreductases, only W- and Se-containing enzymes are found in hyperthermophiles. As the optimum growth temperature decreases, enzymes containing W in association with S are the norm; then, in thermophiles, either W- or Mo-containing enzymes are common; finally, in mesophiles, only Mo-containing enzymes occur. This distribution of these two elements is consistent with their known chemistry. Under the putative hot, anoxic, early-Earth conditions, Mo would have been deposited as MoS_2 in molybdenite, which is insoluble in water and so unavailable. In contrast, W ores, e.g., wolframite and scheelite, always contain tungstate, WO_4^{2-}, which is soluble and would be available to the biosphere. Therefore, if nitrogenase were appearing under these same conditions, Mo would not be available, W would be available but apparently is unable to support N_2 fixation, and so a Fe-only nitrogenase might have been the compromise choice. This view contrasts with the suggestion that nitrogenase arose as an assimilatory cyanide reductase to detoxify local environments (Postgate, 1974). If so, the Mo-nitrogenase would have priority because it is the superior enzyme for reducing cyanide. A third suggestion involves an original optimal nitrogenase from which the three nitrogenases known today developed recently (Postgate, Eady, 1988). This suggestion is supported by: the *nifMBVUS* genes support all three nitrogenases; the three sets of structural genes were likely formed by gene iteration; the heterometal cofactors may be transferred among the proteins; and all three nitrogenases have similar catalytic properties (Newton, 1993).

The classical evolutionary view of the haphazard distribution of diazotrophy among prokaryotes is one of a common ancestral property that was being lost randomly during divergent evolution. Alternatively, the *nif* cluster may be of more recent origin and spreading laterally among diverse prokaryotic genera (Postgate, 1974). However, phylogenies based on either 16S rRNA or *nifH/D* sequences (Hennecke et al, 1985;Young, 1992) indicate that the *nif* genes are established components of their genomes and that they have been present there for as long as those backgrounds have existed.

3. Can Nitrogen Fertilizer Production Support the Growing Population?

After diazotrophy appeared, fixed-N was no longer the limiting nutrient in balanced ecosystems. However, when such ecosystems are perturbed, nutrients recycle geobiochemically and fixed-N usually becomes limiting again. Agriculture is a major and persistent perturbation of natural balanced ecosystems and it produced the predicted result in that global agricultural production is again determined by the availability of fixed-N. The human population may reach 8 billion within 25 years (Gore, 1993). How soon and at what figure the population stabilizes will be determined by social, cultural, religious, political and economic considerations, not by science. What is clearly a scientific question, however, is how to feed and sustain this many people. So far, the combination of the Haber-Bosch process for producing fertilizer ammonia and the Green Revolution, in which high-yielding crop varieties were bred in the context of plentiful fixed-N, has been quite successful in this endeavor. Commercial fertilizer-N currently supports about one-third of the human population. The question now becomes: can fertilizer-N production keep pace with the increasing population? Moreover, should we expect it to do so? If so, what are the consequences of increased fertilizer usage? And what if we are uncomfortable with these consequences? What are our options?

3.1. Effects of Increased Fertilizer Usage

Increased use of fertilizer-N will involve several negative environmental consequences in addition to real financial costs. The first cost will be increased non-renewable energy consumption, mainly for the production of H_2. Industrial fertilizer production presently accounts for about 2% of the worldwide total energy consumption (British Petroleum, 1996). Although newly discovered energy resources have kept up with usage in the recent past, total energy consumption is proceeding at a rate such that all known reserves of oil and gas will be depleted within 50 years. Probably, when oil and gas resources become limited, prices will increase and fertilizer-N manufacturers will use coal and possibly solar power as energy and feedstock sources. The inherent flexibility in feedstock and energy sources will enable fertilizer-N production (and its application to crops) to continue and so will not likely cause of food shortages. Because biological nitrogen fixation could circumvent much of this non-renewable energy cost, it needs to be exploited in every way possible.

The first of the environmental consequences of increased application will be increased nitrate run-off, which will increase contamination of both surface and ground water. Pollution of drinking-water supplies and eutrophication of lakes and streams did not exist in the first half of this century when crop yields and rates of fixed-N application were less than half of today's levels (Bøckman, 1997). Because more fertilizer-N would be supplied, there will be more "left over" fixed-N in roots and also more of the easily mineralized nitrogenous material in the soil to be leached out. This environment "cost", however, is unlikely to be circumvented by using biological nitrogen fixation rather than fertilizer-N. For example, a legume crop leaves significant fixed-N in root residues. Although this residue is beneficial to subsequent crops, it must be mineralized before it can be used and then it can also be leached from the soil. The second environmental impact will be the increased production of nitrogen oxides, which lead to potential atmospheric perturbations. Both nitrous oxide and nitric oxide originate from fertilizer degradation by microbial populations in the soil. Again, substitution of biological nitrogen fixation for fertilizer-N is unlikely to alleviate the problem. Apparently, more nitrous oxide is released from a legume crop than from a reasonably fertilized crop (Eichner, 1990). However, ammonia volatilization should be less from crops with biological nitrogen fixation than from fertilized crops, especially when urea is used. Ammonia volatilization from fertilizer application is a concern because there is no control over where it will be deposited and what its effects will be. So, although biological nitrogen fixation cannot solve all the problems involved with increased fertilizer-N use, it has a major role to play in increasing the fixed-N input into agriculture.

4. Do currently used N_2-fixing systems have the potential to be exploited further?

Besides the very successful, but mature, Haber-Bosch process, there is no obvious abiological technology on the scientific-industrial horizon that offers hope of commercial exploitation in the near-term future. In terms of biological systems, the legume-based symbioses still offer the best hope for increased fixed-N input to agriculture in the short term. Other short-term inputs of fixed-N to rice production, for example, include green manuring with the water fern, *Azolla,* and the semi-aquatic legumes, *Sesbania, Aeschynomeme*, and *Astralagus* and their microsymbionts, and joint culturing with N_2-fixing bacteria, both free-living and informally associated with rice roots. Both input sources have their problems: green manuring involves the added costs of increased labor and land use, seeds and inoculum, and pesticides, whereas informal associations are inefficient in transferring the fixed-N to the crop (Ladha et al., 1997). Some associative symbioses are able to support the growth of some grasses and may, indeed, completely satisfy the fixed-N need of sugar cane (Boddey et al., 1995). The continuing discovery of new associative symbioses will broaden the available avenues for enhancing the delivery of fixed-N to plants.

5. What are Likely Strategies for the Future?

Realistic targets for fixed-N input should have the goals of making agriculture sufficiently productive, sustainable, and more environmentally friendly. One major objective must be how best to supply fixed-N to rice, wheat and corn, none of which are nitrogen self-sufficient. What are the likely targets and input strategies (see, for example, Rolfe et al., 1998)?

5.1. New or Enhanced N_2-fixing Symbiotic, Endophytic and Associative Interactions

A near-term strategy for increased fixed-N input to legumes involves a better match of rhizobial microsymbiont to its host cultivar, earlier initiation and prolongation of symbiotic fixation, microsymbionts that would fix in the presence of fixed-N, and improving utilization of applied fertilizer. Of course, these select strains have to out-compete any indigenous microbial strain. Therefore, the best approach may be to engineer the most competitive rhizobial strains for maximal fixation rather to select the best fixers. These efforts will be assisted by our increasing knowledge of the signaling between plant and microbe(Long, 1992; Dénarié et al., 1993).

With crops other than legumes, increased fixed-N assimilation might come from either modifying naturally-occurring associative symbioses to decrease the loss of fixed-N as it is transferred to the host or from genetically manipulated rhizobia (or others) that establish a symbiotic relationship with cereals. Both approaches suffer from the lack of fundamental information. Even so, these associations may have a significant impact, particularly in developing countries with little fertilizer-N. Establishment of a formal symbiosis would be a major achievement because of the enormous complexity of the symbiotic interaction, which involves the products of more than 100 genes. But, because many of these same genes already exist in plants that do not obviously engage in symbiosis, this target may be achievable, especially if the rhizobial host range is (or is made) broad enough. Already, "nodule-like" structures have been induced on wheat and rice roots in response to an appropriate challenge and these structures have triggered much discussion. However, do we really need a nodule for a stable, cereal-based, symbiotic partnership (de Bruijn et al, 1995)? Again, rather than trying to engineer (or select) a N_2-fixer to invade cereals and form a nodule, it might be easier to engineer a stable endophyte of wheat or rice roots to fix N_2.

5.2. Nitrogen-fixing Plants

A longer-term strategy might involve the development of N_2-fixing plants. To do so, would require success in transferring and then expressing bacterial *nif* genes in plant cells, in supplying adequate energy supplies, and in protecting the nitrogenase proteins from destruction by O_2. Some success has already been achieved. Some *nif* genes have been transferred into plant cells, including plastids. Plastids appear to be the location of choice, because chloroplasts are similar to bacteria in terms of gene organization and expression and because homologues of *nif* genes have been detected therein. Although the plastid could provide the required energy supply via photosynthesis (Ausubel, 1986), it would expose nitrogenase to O_2. This situation may not be serious because O_2-sensitive proteins are known to exist in chloroplasts. Possibly, temporal or spatial separation of nitrogen fixation from photosynthesis could circumvent this concern. Why N_2-fixing plants do not already exist is an intriguing question (Merrick, Dixon, 1984).

5.3. New Catalysts for Producing Nitrogen Fertilizer

An abiological process for fixing N_2 could also improve fixed-N input to crops. If such a process were developed and operated under ambient temperature and pressure conditions, it should overcome the costs of both storage and transportation associated with commercial fertilizer-N. Small-scale, inexpensive, "low tech" systems, especially if driven by a renewable energy resource (wind, water or sunlight), located at the demand site, e.g., in an irrigation stream, and operated when needed, could also lower the non-renewable energy and capital-investment costs, and likely minimize the environmental problems associated with fertilizer-N application.

5.4. Molecular Mechanism of Biological Nitrogen Fixation

If we understood how nitrogenase functioned at the molecular level, we might be able to improve the enzyme's efficiency and increase its fixed-N output. For example, if the obligatory evolution of H_2 during N_2 reduction could be eliminated, up to 25% of the expended ATP could be recovered. Moreover, perhaps the requirement for ATP could be completely circumvented by providing alternatives to the obligatory Fe protein. Possibly, an engineered Fe protein like the L127Δ altered Fe protein (Ryle, Seefeldt, 1996), which is permanently locked in its ATP-bound conformation, might be effective. Can the regulation of both nitrogenase synthesis and activity by NH_3, and possibly O_2, be bypassed? The recently discovered *Streptomyces thermoautotrophicus* nitrogenase indicates that these goals may also be feasible (Ribbe et al, 1997).

6. Summary

As with any technology, progress in nitrogen-fixation research on the practical level is tightly correlated with understanding at the fundamental level. Success at both levels is required if we are to impact food supplies for future generations. Barring a catastrophe, all scenarios of the future encompass a concern of how to feed the increasing human population. While we work to solve our own special pieces of the nitrogen-fixation puzzle, we should not lose sight of the fact that our scientific efforts are ultimately aimed at new or improved methods for the production/ delivery of fixed-N to crops and so for the delivery of food to people.

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SECTION I

BIOCHEMISTRY AND CHEMISTRY

CHEMISTRY AND BIOCHEMISTRY OF NITROGENASE

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In this introduction to the Chemistry and Biochemistry Section of this Congress I shall attempt to take a broad overview of the status of the subject and the advances that will be described. Others will fill in the detail in later papers. Furthermore since I am celebrating working in the field for 30 years I find it interesting to compare our knowledge today with that of 30 years ago:

At that time we knew that nitrogenase consisted of two essential metalloproteins, and that both were damaged by exposure to oxygen and contained iron and acid-labile sulfur. One also contained molybdenum. However the molecular weights of the proteins were not agreed (within a factor of 2!) and thus the metal contents per molecule were also contentious. We had no idea of possible structures of the metallo centres. Mechanistically we knew that MgATP and a low potential reductase were required but the individual roles of the two proteins were unknown. H_2 evolution and the reduction of alternative substrates such as C_2H_2, N_2O, N_3^-,CN^-, and methyl isocyanide had been discovered as had the inhibitors H_2 and CO. Theories on the detailed mechanism of the enzyme were plentiful but unsupported by many facts. One approach to this problem was to study model metal complexes of N_2. 30 years ago several had been synthesised but no characterised N_2 complexes had been reported to be reduced to NH_3.

Today we have made much progress but some of the same problems remain: however we are in a position to address them in much finer detail.

1. Structure

We now have crystallographic structures of the Fe and the MoFe proteins and of the putative, transition state, 2:1 complex formed between them. Solution of the structures of the unique metal-sulfur P clusters and FeMo-cofactor within the MoFe protein has proved difficult in the absence of adequate models but a consensus has now been reached. However higher resolution data are still revealing additional structural details which may have functional significance (Mayer SM et al this volume). Alternative nitrogenases based on V or just Fe in place of Mo have been described (Eady 1996). Structurally these seem to be closely analogous to the molybdenum nitrogenase although the VFe and FeFe proteins include extra small subunits. However an entirely new nitrogenase, from a thermophile, based on molybdenum but apparently with an active site similar to those of the non-nitrogenase molybdenum enzymes and utilising a very novel electron donor system has recently been reported and is described in this volume (Meyer O et al this volume). This discovery may trigger searches for additional nitrogen fixers in unusual environments.

2. Synthesis and biosynthesis of clusters

The structures of the unique P and FeMoco clusters within the MoFe protein present chemists with major synthetic challenges. Neither cluster has yet been synthesised chemically. This is perhaps not surprising since the biosynthesis of FeMoco at least, is known to be extremely complex. Six nif gene products are known to be required and others may be involved in the synthesis. Furthermore there are few clues to the process of biosynthesis of the P clusters. No mutants have been isolated where FeMoco is synthesised but P clusters are not have been isolated (Ruttiman-Johnson et al this volume: Dean D et al this volume).

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3. Mechanism

The Lowe-Thorneley scheme for Mo nitrogenase action proposed in the mid 1980's still forms the basis of our understanding of the reduction of substrates by the enzyme through sequential electron and proton transfers (Thorneley, Lowe, 1985). There is little doubt that FeMoco is the substrate-binding and-reduction site but we still do not know where on FeMoco N_2 is bound or how it is reduced. The current status of this area is summarised by Burgess (this volume) and novel approaches are described in the papers by Thorneley and Watt.

The precise roles of ATP in nitrogenase action are still unclear but site directed mutagenesis approaches, in particular, are allowing new insights into the coupling of energy transduction to complex formation, electron transfer and substrate reduction (Seefeldt et al; Watt et al this volume).

4. Chemistry

During the past 30 years chemists have shown that it is possible to reduce well characterised metal-N_2 complexes to NH_3 at ambient temperature and pressure and have even demonstrated a cyclic electrochemical process for this reduction (see Evans DJ et al, 1999). This chemistry has indicated and continues to indicate a range of possible mechanisms for the enzymic process which are still being investigated with Mo, V or Fe metal complexes where the metals are in environments more closely related to those found in the enzyme (see Davis SC et al, Helleren CN et al this volume). A welcome recent development has been the renewed investigation of the reactivity of FeMoco isolated from its polypeptides (see Shilov:, Pickett et al this volume). These studies are complementary to those on the enzyme and are bringing us much closer to an understanding of the enzyme's active site.

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THE MECHANISM OF MOLYBDENUM NITROGENASE: AN OVERVIEW

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1. Introduction

This article is intended to briefly discuss progress that has been made in our understanding of the mechanism of molybdenum nitrogenase since the appearance of the last comprehensive reviews (Burgess, 1996; Howard, Rees, 1996; Seefeldt, Dean, 1997; Thorneley, Lowe, 1996). A recent review on vanadium nitrogenase appears elsewhere (Eady, 1996). The alternative "iron-only" nitrogenase has been purified in active form but its FeMoco has not been isolated and mechanistic details are not yet revealed (Schneider et al, 1997; Dröttboom et al, 1998; Krahn et al, 1998).

2. Reduction of the Fe protein.

The Fe protein is a 60,000 molecular weight dimer of identical subunits connected through a single [4Fe-4S] cluster. High resolution x-ray structures are now available for free Fe proteins from *Azotobacter vinelandii* and *Clostridium pasteurianum* and for the *A. vinelandii* protein in a complex with the MoFe protein (Howard, Rees, 1996; Schlessman et al., 1998; Schindelin et al., 1997). An alignment of 59 NifH sequences has revealed a core of 140 conserved residues (Schlessman et al., 1998). Until recently the Fe protein's [4Fe-4S] cluster was known to be stable in the same two oxidation states that are utilized by [4Fe-4S] clusters in low potential ferredoxins. These are the oxidized +2 state that formally contains $2Fe^{3+}$ and $2Fe^{2+}$ atoms and the reduced +1 state. For that reason the Fe protein has always been viewed as a one-electron donor to the MoFe protein. In 1994, Watt and Reddy reported that the $[4Fe\text{-}4S]^{1+}$ state of the Fe protein could be further reduced using methyl viologen but not dithionite. The product was reported to have a UV-Vis spectrum similar to the +1 state. No further spectroscopic characterization of that protein has appeared. More recently, the formation of a stable all-ferrous $[4Fe\text{-}4S]^0$ containing Fe protein has been directly demonstrated by Mossbauer spectroscopy (Angove et al., 1997). That all-ferrous state was produced using various low potential reductants (Yoo et al., 1998a). It has an unusual UV-Vis spectrum and pink color and is therefore distinct from the state originally reported. This all-ferrous form of the Fe protein has now been characterized by UV-Vis, CD, MCD, Mossbauer, EPR (S = 4) and x-ray absorption spectroscopies (Angove et al., 1997; Musgrave et al., 1998; Angove et al., 1998; Yoo et al. 1998b). The state can be produced in frozen solution by irradiation and thus the transition does not involve a ligand exchange or protein structural rearrangement (Yoo et al., 1998a). An all ferrous $[4Fe\text{-}4S]^0$ cluster is unprecedented both in model systems with thiolate ligands and in proteins and its formation must rely on a property unique to the Fe protein, perhaps the fact that its [4Fe-4S] cluster is solvent exposed (Morgan et al., 1990). The stability of the all ferrous state suggests that the nitrogenase reaction could function using two- rather than one-electron cycles with the Fe protein shuttling between the +2 and the 0 oxidation states.

The reduction potential needed to produce the well characterized S=4 all ferrous Fe protein is not known but reduction can be accomplished with a variety of chemical reductants with $E^{0'}$ ~-600 mV vs. SHE (Yoo et al. 1998a). In *Klebsiella pneumoniae* the immediate electron donor to the Fe protein is the *nifF* gene product, flavodoxin which likely serves as a one-electron donor reducing the Fe protein only to the +1 level (Thorneley, Deistung, 1988). Thus, if the Fe protein can be reduced by two-electrons in that organism then another protein would be expected to supply the

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second electron. We also find that the purified *nifF* gene product from *A. vinelandii* only reduces the *A. vinelandii* Fe protein to the +1 level. Here, the physiological electron donor(s) is not known because **NifF$^-$** strains still grow well under **N_2-fixing** conditions (Bennet et al., 1998). *A. vinelandii* synthesizes at least two ferredoxins with $\mathbf{E}^{0'}$~-650 mV, Fdl (lismaa et al., 1991) and Fdlll (Gao-Sheridan et al., 1998). FdIII is closely homologous to FdxN which appears to be the physiological electron donor to nitrogenase in *R. capsulatus* (eg. Schatt et al., 1989) and to VnfFd which may be the physiological electron donor for the Fe protein for the vanadium system in *A. vinelandii* (Raina et al., 1993). The actual $\mathbf{E}^{0'}$ values are not known for either protein from *A, vinelandii.*

3. MgATP binding, conformational change and complex formation.

Interactions of the Fe protein with nucleotides have been extensively reviewed elsewhere (Burgess, Lowe, 1996; Howard, Rees, 1996; Seefeldt, Dean, 1997; Thorneley, Lowe, 1996; Schlessman et al., 1998; Schindelin et al., 1997). Briefly, all three oxidation states of the Fe proteins from all sources tested bind 2MgATP (Burgess, Lowe, 1996; Angove et al., 1998) one in each subunit, with positive cooperativity (Lanzilotta et al., 1995; Lanzilotta et al. 1999). There is no structure available of the Fe protein alone with MgATP bound but available information from the structure of the complex now support a "ras-type" binding mode rather then a previously suggested cross-subunit binding mode. MgATP binding induces a large conformational change in the Fe protein that influences the [4Fe-4S] cluster (Burgess, Lowe, 1996). For the 0 oxidation state the same conformational change occurs (Angove et al. 1998). These nucleotide binding reactions have also recently been characterized using **^{31}P** NMR (Miller et al., 1998). The initial conformational change that occurs upon MgATP binding involves the Walker A motif or phosphate-binding loop (P-loop) (Fe protein residues 9-16), two regions corresponding to the Switch I (Fe protein residues 38-43) and Switch II (Fe protein residues 125-135) of the G-protein family and a fourth region around A157 which is unique to the Fe protein (e.g. Seefeldt, Dean, 1997; Schlessman et al., 1998; Schindelin et al., 1997;Bursey, Burgess, 1998). Although the structure of the Fe protein alone in this MgATP bound conformation is not available, x-ray scattering data have established that the conformational change causes the protein to contract on average by 2.0 Å (Chen et al., 1994), the same contraction seen in the new structure of the Fe protein in a complex with the MoFe protein and **$MgADP^{\cdot}AlF_4^-$** (Schindelin et al.,1997). Thus it is likely that the MgATP conformation of isolated Fe protein is similar to that of the Fe protein in the available complex structure. However, these two structures cannot be identical because the Fe protein alone does not hydrolyze MgATP whereas the Fe protein in the complex does.

The main purpose of the large conformational change that occurs on MgATP binding is likely to be to position the protein for the next step in the reaction which is complex formation with the MoFe protein. This in turn allows the MoFe protein to select, from among available Fe protein molecules in the cell, those that have MgATP bound and are therefore able to carry out productive electron transfer. The MoFe protein can be viewed as being composed of two identical halves that do not communicate with each other. For the Mo enzyme each half has one a subunit and one **β** subunit, one FeMo cofactor center, one P-cluster, and one binding site for the Fe protein. For the V and "Fe-only" nitrogenases there is an additional subunit (Eady, 1996). Because the two halves of the MoFe protein function independently both 1:1 and 2:1 Fe protein:MoFe protein complexes are active. Complex formation appears to be rapid, occurring at close to the diffusion controlled limit, and is reversible. There is a great deal of evidence, reviewed elsewhere, that the reaction normally involves the reduced Fe protein in its MgATP conformation (Burgess, Lowe, 1996). Although complex formation is normally transient, a number of different tight complexes have been reported (e.g. Ryle, Seefeldt, 1996; Duyvis et al., 1996; Renner, Howard, 1996; Yousafzai, Eady, 1997).

Recently an x-ray crystal structure of a complex of the Fe protein and MoFe protein from *A. vinelandii,* stabilized by $\mathbf{MgADP^{\cdot}ALF_4^{-}}$, has been refined to 3 A resolution (Schindelin et al., 1997). X-ray scattering data support the idea that this closely represents the complex structure in solution (Grossman et al., 1999).

Large structural changes are evident when the structure of the Fe protein alone is compared to that in the complex. It is not known how many of those changes occurred by binding MgATP (see above) and how many were additional changes that occurred on complexation. The Fe protein conformational change is a ~13° rotation of each monomer toward the subunit interface leading to a more compact structure. This results in a new subunit interface and a nucleotide-binding pocket that now comprises residues from both subunits (Schindelin, 1997; Bursey, Burgess, 1998). The nucleotides are far away from the MoFe protein indicating that all residues needed for subsequent MgATP hydrolysis are contained within the Fe protein. Another change in the Fe protein is that its [4Fe-4S] cluster is completely buried in the Fe protein-MoFe protein interface and is 4 Å closer to the P-clusters contained within the MoFe protein than was predicted by previous docking models (Schindelin, 1997). Unlike the Fe protein, there are no major structural changes within the MoFe protein when the structure of the protein alone is compared to its structure within the complex.

4. Electron transfer from the Fe protein to the P-clusters and MgATP hydrolysis.

In the complex structure, the [4Fe-4S] cluster of the Fe protein is located 14 Å from the P-clusters and 28 Å from FeMoco confirming the long held consensus view that the direction of electron transfer is from the Fe protein to the P-clusters to the FeMoco. Until now the Fe protein has always been viewed as a one-electron donor to the MoFe protein fluctuating between the +2 and +1 oxidation states. The finding that the Fe protein can be reduced to the all-ferrous state and that both electrons can be transferred to the MoFe protein (Watt, Reddy, 1994; Angove et al., 1998) brings up the possibility that the Fe protein could normally serve as a two-electron donor. The P-clusters are double [8Fe-7S] clusters (Peters et al., 1997) that could potentially accept two electrons and two putative electron transfer pathways have been observed in the complex structure between the Fe protein's [4Fe-4S] cluster and the P-clusters (Schindelin, 1997).

Although there is agreement on the direction of electron transfer there is controversy concerning the timing of the electron transfer reactions and two models have emerged. In both models the P-clusters begin in a structurally characterized oxidation state, designated $\mathbf{P^{N,}}$ which is diamagnetic and contains eight $\mathbf{Fe^{2+}}$ atoms (Burgess, Lowe 1996; Peters et al., 1997). In one model $\mathbf{P^N}$ is proposed to first accept an electron from the Fe protein to form a transient super reduced state and is then proposed to transfer an electron to FeMoco (Burgess, Lowe 1996; Angove et al., 1998). Upon complex formation it is known that the $\mathbf{E^{0'}}$ of both the Fe protein and the P-clusters become more negative (Lanzilotta, Seefeldt, 1997; Duyvis et al. 1996). None-the-less the formation of this super reduced state would be thermodynamically unfavorable so that in this model the purpose of MgATP hydrolysis would be to either lower the $\mathbf{E^{0'}}$ of the Fe protein further (19 A away) and/or raise the $\mathbf{E^{0'}}$ of the $\mathbf{P^N/P^{SuperR}}$ couple (33 Å away) to facilitate the electron transfer. Arguments for and against this model are considered elsewhere (Burgess, Lowe 1996; Angove et al. 1998).

In the second model $\mathbf{P^N}$ is proposed to first donate an electron to FeMoco to form a transient oxidized state of the P-clusters and then, the electron hole in the P-clusters would be filled by an electron from the Fe protein. The first reaction would be thermodynamically unfavorable requiring MgATP hydrolysis to either lower the $\mathbf{E^{0'}}$ of the P-clusters (33 A away) or open a physical gate between the P-clusters and the FeMoco (47 Å away). If the Fe protein serves as a one-electron donor than the P- clusters would initially be oxidized to a state that is not yet structurally characterized called $\mathbf{P}^{1+}$. This state can be produced by dye oxidation and can be reduced by dithionite back to the $\mathbf{P}^{N}$ state. When produced by dye oxidation, the $\mathbf{P}^{1+}$ is a mixed S=5/2, S=1/2 system (Tittsworth, Hales, 1993). The $\mathbf{P}^{1+}$ S=1/2 has been observed during turnover in the presence of CO (Cameron, Hales, 1998). If the Fe protein could donate two electrons prior to complex dissociation then the transient oxidized state would be a structurally characterized state designated $\mathbf{P}^{0X}$ (Peters et al., 1997) that can easily be produced by dye oxidation and which exhibits a weak g=12 EPR signal. Arguments for and against this proposal are reviewed elsewhere (e.g. Burgess, Lowe, 1996; Cameron, Hales, 1998; Lanzilotta, Seefeldt, 1997).

The only requirement for MgATP hydrolysis appears to be the formation of the correct complex and there are many situations where MgATP hydrolysis is uncoupled from electron transfer (e.g. Christiansen et al., 1998) However, under normal circumstances, in assays using dithionite, one-electron is transferred from the Fe protein to the P-clusters and two MgATPs are hydrolyzed. Although the all-ferrous Fe protein can transfer two electrons to the MoFe protein it is not yet known if both are transferred prior to complex dissociation (Watt, Reddy, 1994; Angove et al., 1998). If they are, then the MgATP requirement for the reaction would be halved. The order of MgATP hydrolysis and electron transfer has not yet been established. There are data showing that electron transfer can occur in the absence of MgATP hydrolysis under some conditions if the MoFe protein is first chemically oxidized (Angove et al., 1998; Lanzilotta, Seefeldt, 1996a). However, for the first round of electron transfer at least, the MoFe protein begins with both the P-clusters and the FeMoco reduced. Under those conditions there is only one report of very slow Fe protein oxidation occurring without MgATP using a mutant that is stuck in the MgATP-like conformation (Lanzilotta, Seefeldt, 1996b). The reduction of the MoFe protein in that system has not yet been confirmed. It is thus premature to conclude that electron transfer must proceed MgATP hydrolysis or vice versa. The actual energy transduction step in MgATP hydrolysis has also not been established. It could be the initial hydrolysis, phosphate release, the movement of $\mathbf{Mg}^{2+}$ or some other step. It is known that phosphate release occurs after electron transfer but before complex dissociation (Thorneley, Lowe 1996). In addition to the possibility that the energy of MgATP hydrolysis could be used to facilitate forward electron transfer, some or all of the energy could also drive complex dissociation. It was generally believed that the Fe protein could not be re-reduced when in the complex, but more recently that has been accomplished (Lanzilotta, Seefeldt, 1997; Drazhinin et al.,1996a; Druzhinin et al., 1996b; Duyvis et al.,1998).

5. Reactions that occur at FeMoco

The FeMoco center of the MoFe protein is a $[\mathbf{Mo{:}Fe_7{:}S_9{:}homocitrate}]$ cluster of known structure (Howard, Rees, 1996) that can be removed from the protein and studied as an independent entity in N-methyl formamide (Burgess, 1990). When turnover begins, it is in an oxidation state designated $\mathbf{M}^{N}$ which exhibits an S=3/2 EPR signal and appears to have $\mathbf{Mo}^{4+}$, $\mathbf{6Fe}^{2+}$ and $\mathbf{Fe}^{3+}$ (Lee et al., 1997). In turnover experiments, when $\mathbf{M}^{N}$ accepts one electron from the P-clusters it is reduced to a state referred to as $\mathbf{M}^{1-}$, which has integer spin (Burgess, Lowe, 1996). The $\mathbf{E}^{0'}$ for that transition is not known. The isolated FeMoco in the S=3/2 state can also be reduced by one-electron at very low $\mathbf{E}^{0'}$. Kinetics studies have long provided evidence that the MoFe protein could be reduced by more than one electron below the $\mathbf{P}^{N}\mathbf{M}^{N}$ level. At present, however, there is no direct evidence for reduction of the FeMoco center of the MoFe protein below the $\mathbf{M}^{1-}$ **level**.

For substrate reduction to occur FeMoco must accept both protons and electrons. If each electron transfer were followed by a proton transfer then the charge on the cluster would be neutralized facilitating additional electron transfers. This type of protonation controlled gating is known to occur for the [3Fe-4S] cluster of *A. vinelandii* FdI that can be reduced from the all-ferric to the all-ferrous state (Hirst et al., 1998). It is not known where protons reside on the FeMoco site of the MoFe protein prior to being used for substrate reduction. Hydrides have long been proposed to be involved but more recently protonation of the bridging μ_2 sulfides have also been suggested (Burgess, Lowe, 1996;Grönberg et al., 1997;Almeida et al., 1999). There is no direct evidence in support of either proposal, nor can other scenarios be eliminated at present involving homocitrate or other ionizable residues. As is the case for nitrogenase, electron transfer donor-acceptor distances in proteins often exceed 10 Å. Because the equivalent tunneling distances of a proton is less than 0.25 Å, protons must be transported using pathways which are generally composed of water molecules and/or ionizable protein side chains. There is water very near to the FeMoco center of the MoFe protein and the FeMoco is in a very hydrophilic environment with homocitrate and many ionizable residues that could serve as components of a proton transfer pathway. Hydrogen bonding net works are also being considered in this context (e.g. Lee et al.,1998).

The numerous substrate reactions of nitrogenase have recently been reviewed (Burgess, Lowe, 1996) and this discussion will be restricted to recent studies of the physiological substrates and CO which is a noncompetitive inhibitor of all substrate reductions except protons. CO binding to the FeMoco site of the MoFe protein requires turnover conditions, consistent with either a requirement for a conformational change or for the need to first reduce the FeMoco to the M^{-1} state (Tittsworth, Hales, 1993; Lee et al.,1997; Lee et al., 1998;George et al., 1997). CO binding to FeMoco reduced electrochemically has also been demonstrated (Ibrahim et al., 1999). There are at least two binding sites for CO on the FeMoco, both may be iron sites near the “waist” of FeMoco. Both terminal binding modes (for two CO bound) and a bridging mode (for one CO) are likely. The two physiological substrates for nitrogenase are protons and N_2 and the reduction of these substrates has long been believed to require the Fe protein and MgATP. This view now needs reevaluation in light of H_2 evolution by the MoFe protein observed in the absence of MgATP (Yousafzai, Eady, 1999) and because H_2 evolution can occur on isolated FeMoco in NMF (LeGall et al., 1999). To date there is no direct evidence to show how or where N_2 binds on FeMoco and binding through Fe or Mo, end-on, side-on or bridging modes continue to be suggested (Burgess, Lowe, 1996; Stavrev, Zerner, 1998; Shestakov, 1996) with one interesting report proposing that N_2 might bind to Mo by displacement of the histidine ligand in a reaction stabilized by R-homocitrate (Gronberg et al., 1998). At this time, the critical mechanistic questions of how and where N_2 binds and by what mechanism it receives its six-electrons and six-protons remain open for future symposia.

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ROLES FOR NUCLEOTIDES IN NITROGENASE CATALYSIS

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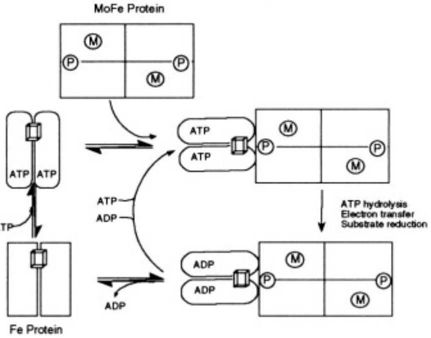
1. Introduction

Nitrogenase catalyzes the reduction of N_2 to NH_3 according to the overall reaction:

$$N_2 + 8H^+ + 16ATP + 8\,e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi.$$

How ATP hydrolysis is coupled to the reduction of N_2 is one of the outstanding open questions about the nitrogenase mechanism, and is the focus of this work. Nitrogenase is a complex metalloenzyme composed of two separable component proteins (Burgess, Lowe, 1996; Dean et al., 1993; Rees et al, 1993). The smaller component (also called the Fe protein, component II, or nitrogenase reductase) is a 64 kDa homodimeric protein with a single [4Fe-4S] cluster covalently linked between the subunits (Georgiadis et al., 1992; Hausinger, Howard, 1983; Howard et al., 1989). The Fe protein contains two nucleotide binding sites, one on each subunit. The Fe protein functions as a specific electron donor to the larger nitrogenase component protein (also called MoFe protein, component I or nitrogenase), in a reaction that is coupled to the hydrolysis of ATP. The MoFe protein contains a Mo-Fe-S-homocitrate containing cofactor called FeMo-cofactor (or M) where substrates bind and are reduced (Shah, Brill, 1977). In addition, the MoFe protein contains an [8Fe-7S] cluster (called the P-cluster), that is likely to be involved in electron transfer between the Fe protein and FeMo cofactor (Chan et al., 1993; Hagen et al., 1987).



The steps of the nitrogenase reaction where ATP is involved include: (i) ATP binding to the Fe protein component, (ii) ATP hydrolysis by the Fe protein-MoFe protein complex, (iii) coupling of ATP hydrolysis to electron transfer and substrate reduction within the Fe protein-MoFe protein complex and, (iv) ATP hydrolysis as a signal for dissociation of the Fe protein from the MoFe protein following electron transfer.

2. Binding of nucleotides to nitrogenase

The Fe protein binds two nucleotides, one on each subunit. It is known that nucleotide binding to the Fe protein results in protein conformational changes that impact the properties of the [4Fe-4S] cluster (Burgess, Lowe, 1996). From a series of site-directed mutagenesis studies (Lanzilotta et al., 1997; Seefeldt et al., 1992; Seefeldt, Mortenson, 1993; Wolle et al., 1992) and x-ray crystal structures of both the uncomplexed Fe protein (Georgiadis et al., 1992; Schlessman et al., 1998) and the Fe protein complexed to the MoFe protein (Rees et al., 1998; Schindelin et al., 1997), it is clear that nucleotides bind some 20 Å away from the [4Fe-4S] cluster (Georgiadis et al., 1992) and the MoFe protein docking surface (Schindelin et al., 1997). Thus, this demands that nucleotides act via long range protein conformational changes. At least two regions of the Fe protein likely to be involved in communication from the nucleotide binding site to the [4Fe-

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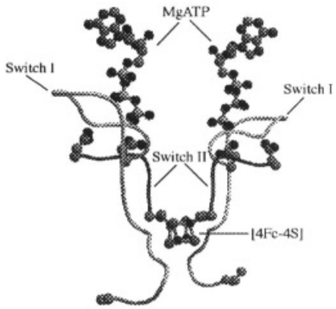
4S] cluster are protein motifs called switch I and switch II. Evidence from both site-directed mutagenesis studies (Lanzilotta et al., 1997; Ryle, Seefeldt, 1996) and an x-ray crystal structure (Schindelin et al., 1997) indicate that both of these switches undergo conformational changes as a result of nucleotide binding to the Fe protein, and thus represent a communication pathway between the nucleotide binding site and the [4Fe-4S] cluster.



3. Roles for nucleotides within the nitrogenase complex

An interesting feature of nitrogenase is that only the Fe protein-MoFe protein complex catalyzes MgATP hydrolysis at detectable rates. This suggests that key residues within the Fe protein necessary for the hydrolysis reaction are not properly positioned until the MoFe protein binds. Essential to understanding how this activation might occur is a consideration of the long distance from the bound phosphate portion of the ATP to the Fe protein surface where MoFe protein binds. This same surface contains the [4Fe-4S] cluster, the properties of which are also influenced over a long distance by the binding of the nucleotide. Given the distance between the nucleotide binding site and the MoFe protein binding surface, it seems reasonable to conclude that protein conformational changes mediate the reciprocal communication between the two regions, probably involving the two switch motifs.

All substrate reduction reactions catalyzed by nitrogenase absolutely require electron transfer from the Fe protein to the MoFe protein coupled to MgATP hydrolysis. While it is possible to deliver electrons to the MoFe protein with small electron transfer agents (e.g., redox dyes) (Watt et al, 1980), to date, none of these has been found to support substrate reduction. This points to a unique role for the Fe protein and MgATP, however, the nature of this role is not well understood. It was generally thought that electron transfer from the Fe protein to the MoFe protein only occurred when coupled with the hydrolysis of MgATP. This model has been challenged by recent observations. The transfer of at least one electron from the Fe protein to the MoFe protein in the absence of any nucleotides has been reported for two different non-dissociating nitrogenase complexes (Chan et al., 1999; Lanzilotta et al., 1996).

Electron transfer reactions from the Fe protein to the MoFe protein have been monitored by several different methods including EPR spectroscopy and absorption spectroscopy. The rates for primary electron transfer from the Fe protein $[4Fe\text{-}4S]^{1+}$ cluster to the MoFe protein have been quantified using stopped flow spectroscopy by monitoring the increase in the absorbance at 430 nm due to the oxidation of the [4Fe-4S] cluster to the 2+ oxidation state (Thorneley, Lowe, 1984). Typically rate constants for primary electron transfer from the Fe protein to the resting state of the MoFe protein are on the order of $100\ s^{-1}$ at 23°C when MgATP is present. In the absence of MgATP or in the presence of MgADP, no detectable electron transfer is observed for the native nitrogenase complexes. At least one role for MgATP in this process comes from analysis of the electron transfer reactions within two different non-dissociating Fe protein-MoFe protein complexes. Within the L127Δ Fe protein – MoFe protein tight complex, a single electron is transferred in the absence of any nucleotides (Lanzilotta et al., 1996). The rate constant for this transfer is low (0.2 s^{-1}), but the addition of MgATP accelerates the rate by approximately 25-fold. For the heterologous *Cp* Fe protein-*Av* MoFe protein tight complex, primary electron transfer is also observed in the absence of nucleotides (Chan et al, 1999). Interestingly, this rate of primary electron transfer is accelerated upon addition of MgATP (over 10^4-fold) to a rate near that observed for the MgATP-dependent reaction in homologous nitrogenase complexes. These results suggest that one role for MgATP in the nitrogenase complex is to accelerate the rate of primary electron transfer. It must also be noted, however, that despite the fast primary electron transfer in the *Cp* Fe protein-*Av* MoFe protein complex, no substrate reduction is observed. Thus, the roles of nucleotides in the overall reaction must involve additional aspects of the mechanism other than just rate acceleration of the electron transfer.

4. Nucleotides influence complex dissociation

Early studies on nitrogenase showed a lag phase for substrate reduction relative to electron transfer and MgATP hydrolysis (Hageman, Bums, 1978). These observations led to the proposal that the Fe protein and MoFe protein go through cycles of association and dissociation following each electron transfer event, with at least two cycles required for the reduction of all nitrogenase substrates (Hageman, Burris, 1978). Subsequent stopped-flow spectrophotometry studies by Thorneley and Lowe on the nitrogenase from *Klebsiella pneumoniae* determined that the slow step in the reaction was dissociation (Thorneley, Lowe, 1983). A recent analysis of kinetic data for the nitrogenase from *A. vinelandii* has challenged this concept, however, and instead suggests that the rate limiting steps involve some undefined slow reactions that take place within the nitrogenase complex prior to dissociation (Duyvais et al., 1998). This question will have to be resolved in future studies. The nucleotide-bound conformation of the Fe protein and the oxidation states of the metal clusters regulate the affinity for binding of the Fe protein to the MoFe protein. One attractive model holds that the hydrolysis of MgATP to MgADP decreases the affinity of the Fe protein for the MoFe protein, favoring dissociation. In support of this idea are recent kinetic measurements by Thorneley and Lowe (Lowe et al., 1995) indicating that complex dissociation does not occur until after release of the hydrolyzed P_i. Likewise, data for variants of the Fe protein suggest that attainment of the MgADP bound state is a signal for dissociation of the two nitrogenase proteins (Lanzilotta et al., 1997; Renner, Howard, 1996; Ryle, Seefeldt, 1996).

Again, protein conformational changes from the nucleotide binding site within the Fe protein to the MoFe protein docking interface are likely to account for changes in protein-protein affinity. In support of this concept, alteration of an amino acid within the Fe protein along one of the signal transduction switches (switch I) has been shown to influence the dissociation of the Fe protein from the MoFe protein even though MgATP hydrolysis proceeds (Lanzilotta et al., 1997; Peters et al., 1994). It has been proposed that complex dissociation allows the exchange of nucleotides and the re-reduction of the Fe protein [4Fe-4S] cluster (Seefeldt, Dean, 1997), though there is some debate as to whether complex dissociation is really a necessary step in the catalytic cycle (Duyvais et al., 1998). Non-dissociating complexes still hydrolyze MgATP, indicating that nucleotides can be exchanged without complex dissociation (Larsen et al., 1995). It was also recently shown that the [4Fe-4S] cluster can be re-reduced within the complex if a reductant with a low enough reduction potential is used, such as flavodoxin (Lanzilotta, Seefeldt, 1997). Yet, even though these non-dissociating complexes can exchange nucleotides and the [4Fe-4S] cluster can be re-reduced, no substrate reduction has ever been observed, supporting the concept that protein dissociation is a integral part of the nitrogenase mechanism.

5. Summary

In summary, it is clear that nucleotides participate at several levels in the nitrogenase reaction. Binding of nucleotides to the Fe protein result in protein conformational changes that are important for subsequent reaction steps. The hydrolysis of ATP by the Fe protein-MoFe protein complex is not absolutely required for electron transfer from the Fe protein to the MoFe protein, but does greatly accelerate this reaction. Finally, the hydrolysis of ATP to ADP and Pi is involved in controlling the dissociation of the Fe protein from the MoFe protein following electron transfer.

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SUPEROXIDE-DEPENDENT NITROGEN FIXATION

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1. Introduction

Streptomyces thermoautotrophicus is a thermophilic, aerobic and obligately chemolithoautotrophic bacterium. It is able to fix dinitrogen with CO or H_2 plus CO_2 as growth substrates. Although CO and H_2 are known as inhibitors of nitrogenase activity, *S. thermoautotrophicus* is able to fix dinitrogen in the presence of these gases. It contains an unusual N_2-fixing system. The structural and catalytic properties of the N_2-fixing system from *S. thermoautotrophicus* were described (Ribbe et al. 1997). It is composed of three enzymes, a heterotrimeric molybdenum-containing carbon monoxide-dehydrogenase (CODH or St3), a heterotrimeric molybdenum-containing dinitrogenase (St1) and a homodimeric manganese-containing superoxide oxidoreductase (St2). Mo-CODH, which supplies electrons to St2, is composed of 87-, 32-, and 17- kDa subunits. St2 reoxidises superoxide radicals and delivers the electrons to St1. St2 is composed of two identical 24 kDa-subunits. St1 reduces nitrogen and is composed of 93-, 33- and 18 kDa subunits (Figure 1).

It is generally accepted that the typical nitrogenase components are very oxygen-sensitive, however both nitrogenase-proteins of *S. thermoautotrophicus* are stable towards O_2. Moreover for the formation of ammonium oxygen is absolutely necessary. As the reduction of N_2 depends obligately on superoxide the N_2-fixing system of *S. thermoautotrophicus* has been named superoxide-dependent nitrogenase (sdn). In contrast to the electronic coupling in known nitrogenase systems via ferredoxin/flavodoxin, dinitrogenase reductase and dinitrogenase, of the superoxide-dependent nitrogenase establishes a molecular coupling via $O_2^{\bullet -}$ (Figure 1). Superoxide operates as a very efficient electron carrier. The N-terminal amino acid sequences of the three subunits of St1 show insignificant sequence similarities with known dinitrogenases, and Southern hybridizations of genomic DNA of *S. thermoautotrophicus* with *nifHDK*-probes derived from *Frankia alnus* and *Klebsiella pneumoniae* revealed no signals. The N-terminal amino acid sequence of St2 was highly similar to Mn-superoxide dismutases of Bacilli but unrelated to dinitrogenase reductases of known nitrogenases. Therefore, our attention was attracted to the genetics and molecular biology of the superoxide dependent nitrogenase from *S. thermoautotrophicus.* In this paper, we have identified St1 as a new addition to the molybdenum-hydroxylase family. The complete primary structure of St2 is highly homologous to Mn-superoxide-dismutases. The identity of *sdnO* being the structural gene of St2 has been demonstrated through the heterologous expression of St2 in *Escherichia coli.*

2. Experimental Procedures

S. thermoautotrophicus UBT1 (DSM 41605, ATCC 49746) was grown as described previously (Ribbe et al. 1997). *E. coli* DH5α (Jessee 1986) and *E. coli* BL21(DE3) (Studier, Moffat 1986) were used as standard host strains for cloning and expression experiments and were grown in LB medium (Sambrook et al. 1989) at 30 °C. The vectors used were pBluescript I KS + (Stratagene, Heidelberg) and pET11a (Novagene, Heidelberg). DNA isolation, Southern hybridizations and DNA sequencing were performed by standard techniques (Sambrook et al. 1989). Recombinant expression of *sdnO* was performed by the pET-expression system and induction with 0.1 mM IPTG. St1 and recombinant SdnO were assayed by the formation of ammonium in a complete nitrogenase

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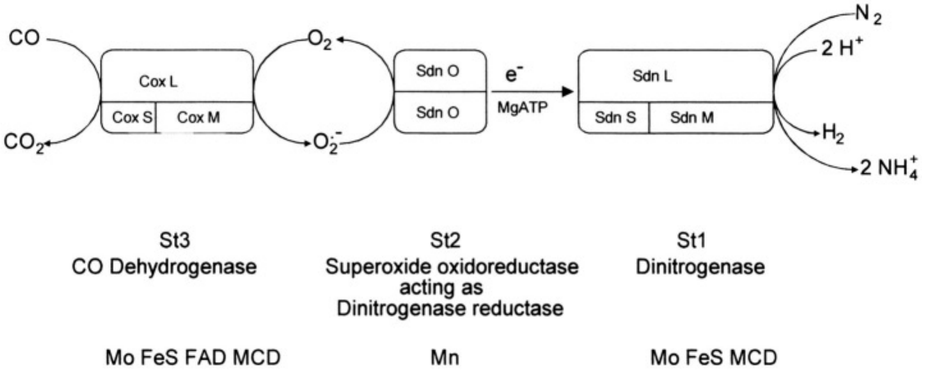

Figure 1. Schematic representation of N_2 fixation in *S. thermoautotrophicus* (Ribbe et al, 1997). Superoxide is produced by CO dehydrogenase through the oxidation of CO and the subsequent transfer of the electrons to O_2. The superoxide is reoxidized by superoxide oxidoreductase that delivers the electrons to a dinitrogenase. The dinitrogenase is capable of reducing N_2 and H^+, but not ethine. MCD, molybdopterin cytosine dinucleotide. The scheme has not been designed to give the correct stoichiometries.

system as described previously (Ribbe et al, 1997). Production and analysis of form A and detection of MCD followed published procedures (Tachil, Meyer 1997).

3. Nitrogenase Structural Genes *(sdn).*

Digested total DNA of *S. thermoautotrophicus* was screened for the three *sdn*-genes coding for St1 using a DIG labeled oligonucleotide probe in Southern hybridizations. The oligonucleotides corresponded to the N-termini of the large (L), medium (M) and small (S) subunits of the St1 protein (Ribbe et al. 1997). The identified nucleotide sequence showed three open reading frames, designated *sdnL, sdnM and sdnS.* The genes are arranged in the transcriptional order (5') *sdnM* (885 bp) → *sdnS* (516 bp) → *sdnL* (2493 bp) (3'). The N-termini of the deduced amino acid sequences and the Sdn-polypeptides were identical. The deduced amino acid sequences of the St1 polypeptides SdnM, SdnS and SdnL show high similarity to molybdenum containing hydroxylases (Figure 2) and only negligible similarity to dinitrogenases. Digested genomic DNA of *S. thermoautotrophicus* was used in Southern hybrizations with labeled oligonucleotide probes derived from the N-terminus of the St2 protein. On a subcloned fragment an open reading frame of 624 nucleotides designated as *sdnO* was identified. The N-termini of the deduced amino acid sequence and the SdnO-polypeptide were identical in the first 21 positions. *sdnO* is not part of the *sdnMSL*-cluster and is surrounded by genes which have no known function in nitrogen fixation.

3.2. SdnM

SdnM reveals significant similarity to the matching sequences in other Mo-hydroxylases (Figures 2, 3). With few exceptions most molybdenum-containing hydroxylases are flavoproteins (Wootton et al. 1991) and it is generally assumed that FAD is associated with the medium sized subunit or domain. That the M subunit is the flavoprotein is apparent from the high resolution crystal structure of CODH of *O. carboxidovorans* (Dobbek et al. 1999). In addition, the absence of the medium sized subunits or domains corresponds with the lack of FAD (Romao et al. 1995; Lehmann et al. 1995). The coincidence of FAD and medium sized subunit is discontinued in the Stl-protein from *S. thermoautotrophicus,* where FAD is absent.

3.3. SdnS contains motifs indicative of type I and II [2Fe:2S]

SdnS reveals significant sequence similarities to the 2Fe:2S-containing subunits or domains of members of the sequence family of molybdenum hydroxylases (Figures 2, 3). The motif C^{39}-X_4-C^{44}-X_2-C^{47}-X_{11}-C^{59} at the N-terminus of SdnS is homologous to the [2Fe:2S] type II binding site of bacterial and chloroplast-type ferredoxins. The C-terminal motif H^{94}-X_3-C^{98}-X_2-C^{101} and C^{133}-X-C^{135} is indicative of the unusual type I [2Fe:2S] center of Mo-hydroxylases. Obviously, SdnS is the iron-sulfur protein of St 1.

3.4. SdnL contains a molybdenum cofactor binding motif

The large sized subunit SdnL reveals significant sequence similarities to the large subunits or domains of members of the sequence family of molybdenum hydroxylases harboring the Moco (Figures 2, 3). The sequence ^{248}GGGFGNKV of SdnL corresponds closely to the conserved sequence GGGFGG of xanthine dehydrogenase (Hughes et al. 1992), which has been shown to be involved in binding the molybdenum cofactor by mutational analysis in *D. melanogaster.* This motif and further segments were structurally identified in CODH from *O. carboxidovorans* (Dobbek et al., 1999) and in Mop (Romao et al., 1995) as amino acids contacting the molybdopterin-cytosine-dinucleotide cofactor. These segments are highly conserved in molybdenum-containing hydroxylases in terms of sequence, relative location within the protein, and amino acid environment.

These assignments agree with the identification of Mo-MCD in Stl. The fluorescence spectrum reveales that Stl contains at least molybdopterin as a cofactor. The spectrum showed excitation maxima at 281, 313 and 397 nm and the emission maxima by 463 nm. The spectrum was almost identical to the typical fluorescence spectrum of form A from CODH of *O. carboxidovorans* (Tachil, Meyer 1997). The preceding analysis revealed that the nature of the nucleotide present in the molybdopterin cofactor is molybdopterin cytosine dinucleotide [di(cam)], since the spectrum of [di(cam)] derivate was similar to the [di(cam)] derivate of CODH of *O. carboxidovorans.* The absorbance maxima of [di(cam)] derivate of Stl were at 279 and 371 nm and A_{279}/A_{371} ratio was 3.27, which is similar to the absorbance ratio of MCD containing xanthine dehydrogenase of *Veillonella atypica* (Gremer, Meyer 1996).

3.5. SdnO

The homology of SdnO to Mn-SODs is about 80% (Figure 4). The similarity to dinitrogenase reductases is not significant. The amino acid sequence of SdnO carries the motif ^{164}DVWEHAY^{170}, which is the Mn- and Fe-superoxide dismutase signature D-X-WEH-(S,T,A)-(F,Y) (Parker, Blake 1988), as well as the other ligands involved in the Mn coordination (His^{26}, His^{81}; Figure 3). As shown by Ribbe et al. (1997), it is not possible to replace St2 by Mn- or Fe-SODs in activity assays. Moreover, these proteins inhibit ammonium formation under assay conditions. SODs catalyse the disproportion of superoxide anion radicals to oxygen and peroxide and thus scavange the radicals, which are the motive force of N_2 reduction.

3.6. Heterologous expression of sdnO

The yield of recombinant SdnO in *E. coli* was 30-40% of the total cytoplasmic protein. Recombinant SdnO was entirely soluble and fully assembled as a Mn-containing homodimer. It was purified to homogeneity employing a heatstep at 65°C as the most efficient step. Purified SdnO was catalytically active and able to use superoxide anion radicals as substrate and to transfers the electrons to St1, driving the formation of ammonium (Table 1).

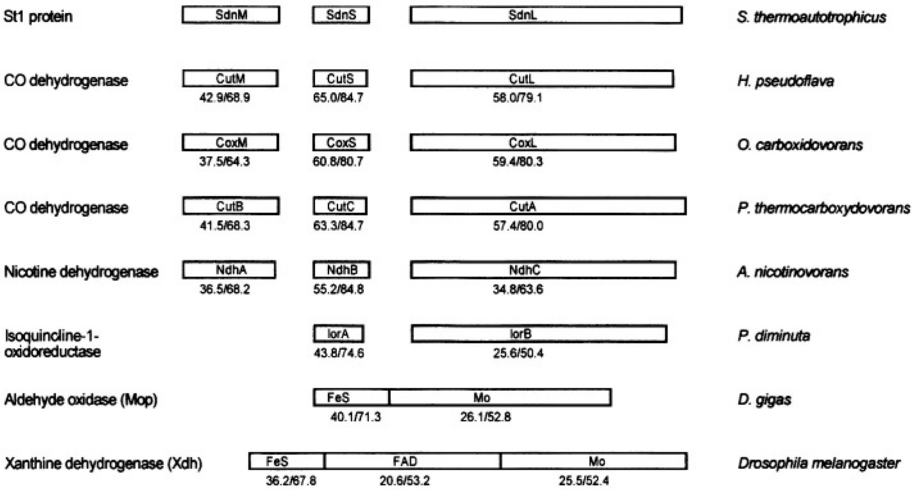
St1 protein
SdnM
SdnS
SdnL
S. thermoautotrophicus
CO dehydrogenase
CutM
CutS
CutL
42.9/68.9
65.0/84.7
58.0/79.1
H. pseudoflava
CO dehydrogenase
CoxM
CoxS
CoxL
37.5/64.3
60.8/80.7
59.4/80.3
O. carboxidovorans
CO dehydrogenase
CutB
CutC
CutA
41.5/68.3
63.3/84.7
57.4/80.0
P. thermocarboxydovorans
Nicotine dehydrogenase
NdhA
NdhB
NdhC
36.5/68.2
55.2/84.8
34.8/63.6
A. nicotinovorans
Isoquinoline-1-oxidoreductase
IorA
IorB
43.8/74.6
25.6/50.4
P. diminuta
Aldehyde oxidase (Mop)
FeS
Mo
40.1/71.3
26.1/52.8
D. gigas
Xanthine dehydrogenase (Xdh)
FeS
FAD
Mo
36.2/67.8
20.6/53.2
25.5/52.4
Drosophila melanogaster

Figure 2. **Comparison of Sdn-polypeptides with other molybdenum hydroxylases. The Sdn-polypeptides are compared with the closely related polypeptides or domains of molybdenum hydroxylases of different organisms. The bars are proportional to the size of the polypeptides or domains. The numbers refer to identities/similarities relative to the Sdn-polypeptides.**

4. Discussion

The structural genes *sdnM, sdnS* and *sdnL* of *Streptomyces thermoautotrophicus* were cloned and sequenced. We conclude from the following results that *sdnM, sdnS* and *sdnL* code for the St1-protein: (i) the predicted molecular masses of the polypeptides SdnL, SdnM and SdnS correspond to the molecular masses of the large, medium and small sized subunit as estimated by SDS-PAGE (Ribbe et al. 1997); (ii) the deduced amino acid sequences of the polypeptides totally match the N-termini, as determinated by Edman degradation (Ribbe et al. 1997). The sequence alignments revealed negligible similarities with dinitrogenases. However, the SdnM-, SdnS- and SdnL-polypeptides showed significant homologies to molybdenum-containing hydroxylases, the highest similarities were observed with carbon monoxide dehydrogenases (CODHs) of various bacteria.

In *S. thermoautotrophicus* CODH is the key enzyme of the chemolithoautotrophic utilization of carbon monoxide (Gadkari et al. 1992). We sequenced *coxS,* the structural gene of the FeS-subunit of CODH (unpublished data). The similarity between *sdnS* and *coxS* is 79.3%, the identity 59.8%. In the Sdn-polypeptides FAD is absent (Ribbe et al. 1997). The CODH from *S. thermoautotrophicus* has no nitrogenase activity and *vice versa* (Ribbe et al. 1997).

Sequence similarities of SdnS with small subunits of Mo-hydroxylases were clustered in two regions (Fig. 3), the N-terminal domain harbouring the motif indicative of type II [2Fe-2S]-cluster and the C-terminal domain harbouring the motif indicative of type I [2Fe-2S]-cluster. The amino acid consensus sequence for a molybdenum cofactor binding site is present on SdnL (Fig. 3). This finding is confirmed by following results: (i) identification of pterin as cofactor; (ii) analysis of molybdopterin cytosine dinucleotide; (iii) presence of Mo in the purified protein in stoichiometric amounts (Ribbe et al. 1997).

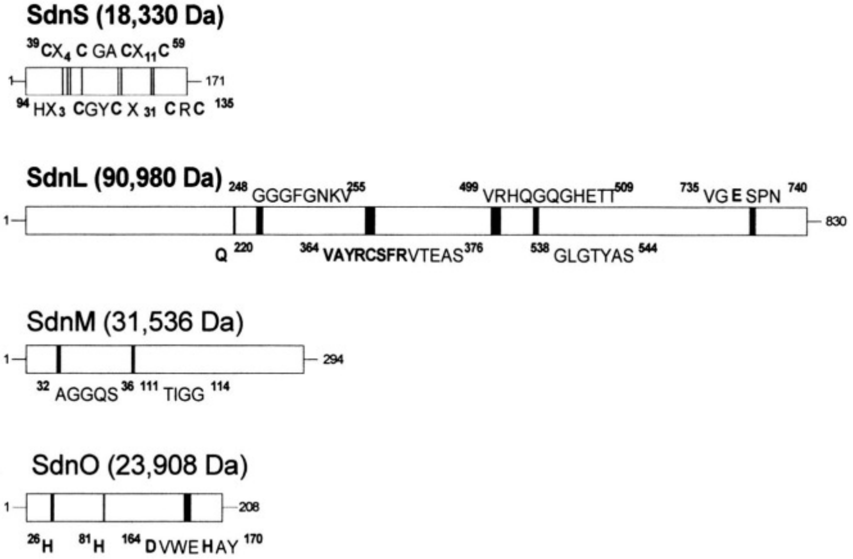
SdnS (18,330 Da)
39CX4 C GA CX11C 59
1
171
94 HX3 CGYC X 31 CRC 135
SdnL (90,980 Da)
248 GGGFGNKV255
499 VRHQGQGHETT509
735 VG E SPN 740
1
830
Q 220
364 VAYRCSFRVTEAS376
538 GLGTYAS 544
SdnM (31,536 Da)
1
294
32 AGGQS36 111 TIGG 114
SdnO (23,908 Da)
1
208
26H
81H
164 DVWEHAY 170

Figure 3. Signatures on the Sdn-polypeptides indicative of the molybdopterin molybdenum cofactor, 2Fe:2S-centers and SOD-type Mn. SdnS, the bold letters represent cysteine ligands of the 2Fe:2S-centers. SdnL, the motif ^{248}GGGFGNKV^{255} refers to the binding site of the molybdenum cofactor. The other segments bind MCD. The motif ^{364}VAYRCSFR^{371} is the active site loop of CO dehydrogenases. SdnM, the amino acids are responsible in binding of pyrophosphate. SdnO, ^{26}H, ^{81}H, ^{168}H and ^{164}D are the presumed ligands of Mn.

The high resolution structure of CODH from *O. carboxidovorans* (Dobbek et al. 1999) and of aldehyde oxidoreductase (Mop) from *D. gigas* (Romao et al. 1995) have been solved and afford an insight into the active site of these molybdenum hydroxylases. Both harbour [2Fe-2S] centers of type I and type II and a MCD-molybenum cofactor. However, Mop consists of a single polypeptide chain and lacks FAD whereas CODH is a heterotrimeric flavoprotein. The MoFeS-protein of the superoxide dependent nitrogenase compares to Mop and CODH with respect to the presence of the three subunits, Mo, MCD and [2Fe:2S] of type I and type II. Differences pertain to the absence of FAD in St1 and that it is only a monomeric heterotrimer. Analogously to CODH the MCD-molybdenum cofactor is probably buried at the center of the SdnL-polypeptide and is surrounded by hydrogen bonds. As in CODH ^{240}Q, ^{388}C and ^{763}E in SdnL ^{220}Q, ^{368}C and ^{737}E are the residues responsible for tying the cofactor in the protein matrix. The unique active site loop ^{384}VAYRCSFR on the L subunit of *O. carboxidovorans* CODH is present on the L subunit of St1 as ^{364}VAYRCSFR as well. In CODH ^{388}C plays an essential role in the reactivity of the enzyme. This amino acid residue is modified as S-selenylcysteine (Dobbek et al. 1999) and may bind CO.

All known nitrogenases require MgATP. The N_2-fixing system from *S. thermoautotrophicus* indicates a very high efficiency with a MgATP/N_2 ratio of 4 (Ribbe et al. 1997). In contrast, nitrogenases from other sources have a theoretical ratio of 16. It is generally accepted that the two components of nitrogenases, the Fe-protein and MoFe-protein, associate in a transient complex (Schindelin et al. 1997). Binding of MgATP causes substantial conformational changes in the protein, which are necessary to build the complex. Exclusively in this arrangement, ATP-hydrolysis occurs, coupling energy consumption to electron transfer from the Fe-protein via the P-cluster to the FeMo-cofactor. The residues involved in ATP-binding and -hydrolysis are highly conserved. They are arranged in Walker motif A (GxGx$_2$GKS), Walker motif B (Dx$_2$G) and three further regions. In the superoxide dependent nitrogenase the combination of this motifs are absent. Nevertheless we found glycine-rich regions and fivefold the motif Gx$_2$G (^{25}GYDG, ^{110}GTIG, ^{140}GPQG, ^{205}GTDG, ^{289}GKVG) and the Walker motif B (^{53}DING). The amino acid sequences ^{32}AGGQS and ^{111}TIGG on SdnM are very similar to those on CoxM (^{32}AGGHS, ^{111}TIGG) concerning the succession and relative position within the polypeptide. The high resolution structure of CODH shows that the

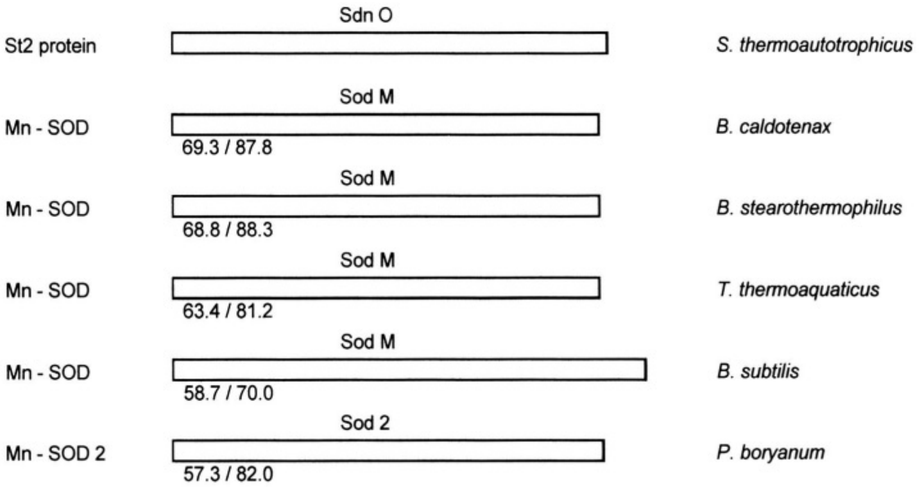

Figure 4. Comparison of the SdnO-polypeptide with Mn-superoxide dismutases. The SdnO-polypeptide is compared with the closely related Mn-SODs from different organisms. The bars are proportional to the size of the polypeptide. The numbers are given as identity and similarity, respectively.

residues play an important role in binding the adenine and pyrophosphate moiety of FAD, whereas the isoalloxazine ring is only weakly bond to the protein matrix (Dobbek et al. 1999). The reactivity of deflavo-CODH further extends these result (Gremer et al., submitted). This CODH-species is able to bind stoichiometric amounts of ADP, substoichiometric amounts of ATP, but not at all riboflavin and AMP. It shows no affinity to CDP or GDP. The findings support the thinking that ATP-binding probably occurs on SdnM.

The structural gene *sdnO* was cloned and sequenced. The translated amino acid sequence revealed high similarity to Mn-SODs as shown in Figure 4. The multiple sequence alignment of SdnO and 94 superoxide dismutases shows 5 amino acids (^{86}P, ^{87}M, ^{100}F, ^{133}V, ^{142}T), which are only present in SdnO. The gene was heterologously overexpressed in *E. coli.* The completely assembled recombinant SdnO was soluble and catalytically active as electron donor for St1 (Figure 1). Mn- and Fe-SODs from other organisms are not able to function as dinitrogenase reductase. Although recombinant SdnO shows very high similarity with Mn-SODs, we could show that it is functionally different in not being able to catalyse the reduction of $O_2^{\bullet -}$ to O_2^{2-}.

Table 1. The activities of purified recombinant SdnO

	Formation of NH_4^+ (nmole NH_4^+ h^{-1} mg^{-1}) 1)	Oxidation of superoxide (units mg^{-1}) 2)
recombinant SdnO + St1	266	-
recombinant SdnO	0	114
St1	0	0
St2	0	104
St1 + St2	109	-
St1 + control 3)	0	-
control 3)	0	0

1) Ribbe et al. 1997

2) PMS/NADH as source of superoxide as described (Ewing, Janero, 1995)

3) cytoplasmic fraction of *E. coli* BL21(DE3) pET11a without *sdnO* after heatstep

The nitrogen-fixing system of *S. thermoautotrophicus* has the following specific properties:
(i) it requires oxygen; (ii) it is insensitive towards CO and H_2; (iii) the cofactor is Mo-MCD; (iv) SdnM, S, L are not related to NifHDK, VnfHDGK and AnfHDGK; and (v) reduction of N_2 requires only 4 mole MgATP. The stability of the nitrogenase components towards O_2 makes them ideal candidates for the transformation into plants.

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CHEMISTRY AND BIOCHEMISTRY OF NITROGENASE (PART 1)

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It is clear from a comparison of the reactivities of the isolated FeMo-cofactor with those of the MoFe protein that understanding the role of the protein environment of the cofactor is essential to understanding how nitrogenase functions. It is very likely that the immediate environment (e.g. H-bonding and charge interactions with protein side chains, steric constraints) of not only the FeMo-cofactor but also the adjacent P cluster change during turnover due to protein conformation changes consequent on ATP hydrolysis events occurring on the Fe protein. Evidence to support this hypothesis is beginning to emerge from the high resolution X-ray structure of the *Klebsiella pneumoniae* MoFe protein (see Mayer et al this volume) which shows that changes in the oxidation level of the P cluster are coupled to movement of the alpha 7 helix which, by comparison with the Avl-Av2 structure (Schindelin et al 1997), is located at the interface with the Fe protein in the nitrogenase complex. High-resolution X-ray structures of the MoFe-protein - Fe protein complex at various oxidation levels and in various conformations (stabilised by MgATP, MgADP-Pi, MgADP analogues) are clearly needed. The mechanism of the coupling of ATP hydrolysis to electron transfer reactions and substrate protonation is still poorly understood and further complicated by the intriguing results of Watt et al (see this volume) with T i (III) citrate as the reductant which show that ATP:2e values close to 2 can be obtained under certain conditions. Although this result is ascribed by Watt et al. to the ability of the all ferrous form of the Fe protein to transfer two electrons to the MoFe protein, this needs to be further investigated by a range of time resolved spectroscopic and chemical/freeze quench techniques under single turnover conditions.

Thorneley et al (this volume) have shown the potential of time resolved infra-red spectroscopy to detect and characterise small molecules bound to functioning nitrogenase. The technique complements other time resolved spectroscopies such as stopped-flow spectrophotometery and rapid-freeze epr spectroscopy which have principally been used to monitor changes in oxidation levels of the three types of cluster present in the Fe protein - MoFe protein complex. The IR experiments are technically difficult to perform and data extraction demanding because of the small amplitudes of the bands which are superimposed on high backgrounds associated with the nitrogenase proteins and the aqueous medium. The interpretation of these data is complicated by the apparent multiplicity of sites on the FeMo-cofactor that could involve terminal and/or bridging modes of reducible substrate molecule binding as judged by the current data for the inhibitor CO. Interpretation is further complicated by time dependent changes in the oxidation level of the FeMo-cofactor shown by parallel epr studies. An advantage of IR spectroscopy is that it monitors small molecule binding to all oxidation levels of the FeMo-cofactor whereas techniques such as ENDOR can only monitor CO binding to paramagnetic states (Lee et aL 1997). The reference data base of IR frequencies provided by chemical model systems (see Richards this volume) and electrochemical / IR studies on isolated FeMo-cofactor at various oxidation levels (see CJ Pickett this volume) are essential to the interpretation of the IR data obtained with functioning nitrogenase. Time resolved IR spectroscopy also has the potential to directly monitor the energy transduction events associated with ATP hydrolysis not only by observation of the phosphate

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ester IR bands but also of the alpha and beta amide bands of the proteins which are sensitive to changes in conformation. Dilworth et al reported on two MoFe protein mutants. Neither of these is able to effectively reduce nitrogen although both are able to reduce protons and acetylene at high levels. Even though nitrogen is not an effective substrate for either protein, preliminary evidence indicates that N2 is able to bind both proteins. Of interest is that one altered protein supports N2-dependent HD formation while the other apparently does not. If correct this means that the simple binding of N2 to the active site is not sufficient to elicit HD formation.

In addition to its role in the catalytic process, the biogenesis of FeMo-cofactor has been the subject of considerable investigation for the past two decades. Although the number of players involved in this complex process has been known for some time, and some insight concerning the sequence of events has emerged, details concerning the composition and structures of the chemical intermediates involved in FeMo-cofactor formation is not yet known. One difficulty has been the absence of a suitable radiolabled, stable, form of Mo. To circumvent this problem Ludden and coworkers have begun the analysis of the VFe-cofactor (homolog to the MoFe-cofactor) with great success (see Ruttimann Johnson et al). These and subsequent studies have indicated that the VnfX (or NifX) protein serves as carrier of a cofactor intermediate. Considering the small size of the VnX protein such studies provide considerable promise for elucidation of a high resolution structure of a protein-bound cofactor intermediate. There has also been some advance in the understanding of the mobilization of iron and sulfur for nitrogenase metallocluster assembly. It was previously reported that NifS is a cysteine desulfurase that probably supplies the inorganic sulfur necessary for nitrogenase metallocluster assembly. It has now been shown that NifU binds iron that is destined for iron-sulfur core formation and that it provides a site for the assembly of a 2Fe-2S unit that serves as a precursor for the nitrogenase metallocluster cores (see Dean et al). There is also mounting evidence that the mechanism for activation of iron and sulfur for nitrogenase metallocluster formation could represent a general mechanism for maturation of Fe-S cluster-containing proteins.

In spite of significant advances in our understanding of the enzymology of nitrogenase catalysis over the past several years the most significant questions concerning where and how substrates are bound to the active site remain unanswered. The major challenge for cluster assembly studies will be to elucidate the chemical composition and structures of cofactor intermediates.

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A 1.6A RESOLUTION X-RAY CRYSTALLOGRAPHIC STUDY OF *KLEBSIELLA PNEUMONIAE* MoFe PROTEIN, Kp1

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1. Introduction

Within the $\alpha_2\beta_2$ MoFe proteins each $\alpha\beta$ half contains a pair of unique metal sulfur clusters: an iron-sulfur P cluster and an iron-molybdenum sulfur cofactor (FeMoco). Peters et al (1997) reported the structures at 2A resolution of the P cluster of Av1 as an 8Fe-7S cluster in both oxidized and reduced states. The reduced P cluster could be described as two 4Fe-4S cubanes fused at one corner giving rise to a central sulfur atom with distorted octahedral coordination to six iron atoms, as first proposed by Bolin for Cp1 at 2.2A resolution (Bolin et al 1993). The P cluster is bound pseudo-symmetrically at the α/β subunit interface by three cysteine residues from each subunit. On oxidation, two iron atoms in one half of the P cluster move away from the central sulfur and form new covalent bonds with a main chain nitrogen and a serine hydroxyl group respectively. FeMoco can be described as constructed from 4Fe - 3S and Mo-3Fe-3S partial cubanes bridged by three sulfur atoms. It is bound within the α subunit approximately 10 A from the protein surface in a largely hydrophilic pocket with only two protein ligands; Cys to the unique tetrahedral iron atom and His to the molybdenum atom. In addition, R homocitrate binds the molybdenum atom through its hydroxyl and a carboxyl group to give full octahedral coordination.

2. Results

The above description of the metal sulfur clusters within the MoFe protein has taken some time to develop, earlier descriptions (Kim, Rees 1992: Chan et al 1993) being erroneous in some aspects. We have now determined the X-ray crystal structure of Kp1 at a resolution of 1.6A with the protein in at least two different oxidation states and can confirm the above description of the P cluster and FeMoco structures. At this resolution it is possible to completely resolve the atoms in the clusters thus providing accurate interatomic distances for these metal centres. In the final stages of refinement all geometrical restraints were turned off to reduce bias. Our analysis has revealed additional features of the MoFe protein structure which may have functional significance. For the purposes of this article we will concentrate on the most significant differences between the Kp1 and Av1 structures. A more detailed description is given elsewhere (Mayer et al 1999).

2.1 FeMoco centres

One striking difference between the FeMoco centres of Av1 and Kp1 is the semi-reduced/oxidized mid point potential which is -47mV for Av1 and -180mV for Kp1 at pH8. Comparison of the structures indicates that this difference in potential may be associated with a subtle difference in the position of Arg α95 (α96 in Av1) which, in Av1, can form a weak hydrogen bond to a sulfur on the equator of FeMoco, whereas in Kp1 the orientation of the side chain does not allow this interaction.

2.2 P clusters

Close examination of the data for Av1 reveals that in the "reduced" P cluster the distance between Fe6 and the central sulfur S1 is relatively long (2.60 or 2.92A in the two halves of the molecule) whereas in Kp1 this distance is 2.47A which compares favorably with the overall average Fe-S1 length of 2.45±0.5A. Since the reduced structures were determined on protein which was undoubtedly oxidized during crystallization and then reduced by dithionite prior to structure

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determination it seems likely that the Av1 structure represents an incompletely reduced P cluster and may indicate that the Fe6-S1 bond is the last to form on reduction. With Kp1 we have also collected data on a crystal form which appears to be intermediate between the fully reduced and oxidized P clusters. These data can be fitted assuming half occupancy by the Fe5 and Fe6 iron atoms of their positions in the fully oxidized or fully reduced P cluster. None of the other iron or sulfur atoms in the P cluster change position during this transformation.

Since the P cluster spans an inter-subunit boundary, it is well placed to transmit redox-mediated conformational changes to the rest of the protein. In Kp1 Ser β90 (β92 in Av1) is a totally conserved residue which lies adjacent to the P cluster. In the dithionite-reduced and mixed structures two conformations of this side chain can be observed. In one the O_γ hydrogen bonds to a water whilst in the other it bonds to a different water molecule and in addition is only about 3A from a sulfur atom of the P cluster possibly forming a weak hydrogen bond to it. However, in the two-electron oxidized structure, only the first of these conformations is apparent and only this one is seen in Av1 and Cp1 models. This mobility of Ser β90 in Kp1 makes it a candidate for the pH dependence of the $P^{semi\text{-}ox}/P^{ox}$ transition observed for Av1 (Lanzilotta et al 1998) and thus may indicate a possible role in coupled electron and proton transport. Alternatively this interaction may be important in stabilizing the highly reduced (all ferrous) P cluster. It may not have been seen in the Av1 model since (as noted above) this does not appear to be completely reduced.

2.3 Protein structure

A further difference between the oxidized and reduced Kp1 structures is the position of helix α7 in the β chain comprising residues Glu β198 to Phe β123. In Av1 this helix forms part of the Av2 docking surface in the transition state complex (Schindelin et al 1997). This “docking helix” occupies roughly the same position in the oxidized and mixed structures but in the reduced Kp1 model is displaced towards the expected position of the 4Fe-4S cluster of the Fe protein in the transition state complex and thus its movement may be functionally significant.

In addition we have detected a non-proline *cis*-peptide bond between Trp α251 and Ser α252 within 10A of FeMoco. It is possible that this *cis*-peptide has been overlooked in other MoFe protein structures at lower resolution and it is tempting to speculate that Ser α252 routinely undergoes *cis/trans* isomerization acting as some kind of conformational switch. This switch could be involved in proton and/or electron transfer or have some role in FeMoco insertion.

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4. Acknowledgements

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ROLES OF VnfX AND NifX IN FeV-CO AND FeMo-co SYNTHESIS IN *AZOTOBACTER VINELANDII*

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Azotobacter vinelandii harbors three genetically distinct nitrogenase systems: a molybdenum-containing nitrogenase, a vanadium containing nitrogenase and a iron-only nitrogenase, encoded by the *nif, vnf,* and *anf* genes, respectively (Eady 1991). Nitrogenases contain a unique iron-sulfur-heterometal cofactor in their active site (the iron-molybdenum-cofactor, FeMo-co for the *nif*-encoded nitrogenase and the iron-vanadium cofactor, FeV-co for the *vnf*-encoded nitrogenase). The *nif, vnf* and anf regulons code for the structural components of nitrogenases and also for auxiliary proteins required for the biosynthesis of the cofactors and their insertion in the cofactorless nitrogenases. Among the genes known to be involved in FeMo-co synthesis are *nifB, nifN, nifN, nifV* and *nifH.* Of these, *nifB* and nifV are also required for FeV-co synthesis, since strains with mutations in these genes are not capable of vanadium-dependent diazotrophic growth (Joerger et al. 1988, Kennedy et al. 1992). Analogs of the *nifE, nifN* and *nifH* genes exist in the *vnf*-system *(vnfE, vnfN* and *vnfH,* respectively). The roles played by these gene products in the biosynthesis of the cofactors are beginning to be understood, but the complete biosynthetic pathway is unestablished. We report that the products of *nifX and vnfX are* involved in the synthesis of FeMo-co and FeV-co.

An iron and vanadium-containing cluster accumulates on VnfX. When a mutant strain unable to synthesize the structural proteins of the vanadium nitrogenase (CA11.1 D*nifHDK*D*vnfDGK1::spc*) was grown in a medium depleted of Mo and containing 49V (radioactive), one radiolabeled protein band was observed by using phosphorimaging of anoxic native gels (Fig. 1, lane 2, labeled VnfX). 49V did not accumulate on this protein in strains that are impaired in FeV-co synthesis (CA117.3, D*nifHDK*D*nifB* and CA11.8, D*niJHDK*D*vnflf*) or when the cells were grown in the presence of ammonium (not shown). 49V accumulated on the *vnf*-dinitrogenase in a strain which is a "wild-type" for the *vnf*-nitrogenase system (CA12 (*DnifHDK*)) (Fig. 1, lane 1). The radiolabeled protein present in extracts of *A. vinelandii* CA11.1 was purified and identified as VnfX, based on its N-terminal sequence identity to the amino acid sequence predicted from the *vnfX* gene (MIKVAFASN). EPR analysis of dithionite-treated (as isolated) VnfX was consistent with a $S=3/2$ species that was of maximum observed intensity at 4.8 K (Rüttimarm-Johnson et al. 1999). The ground state spin and spectral line shapes resembled those of isolated FeV-co (Smith et al. 1988), the reduced *vnf*-dinitrogenase of *A. chroococcum* (Eady et al. 1987), and a synthetic $[VFe_3S_4]$ cubane cluster (Carney et al. 1987). Metal analysis revealed that there are 8±1 mols of Fe and 0.5 mols of V bound per mol of VnfX. The EPR and metal analyses are consistent with a VFe_7S_x cluster bound to VnfX. However, it does not appear that FeV-co itself accumulates on VnfX, as V- and Fe-containing VnfX was not able to donate ^{49}V label to FeV-co-deficient dinitrogenase (VnfDGK) in different conditions tested. The low level of V (0.5 mole/mole VnfX) may indicate that some V was lost during purification or that a fraction of the cluster on the isolated VnfX had not yet acquired an atom of V.

VnfX and NifX (as purified from nifB strains) are able to bind NifB-co. Immunoblot analysis of anoxic native gels of partially purified VnfX and NifX showed that these proteins can exist as two electrophoretically distinct species, depending on whether they are purified from a *nif*B$^-$ or a *nif* B$^+$ strain. VnfX that had the V and Fe containing cluster bound (from *A. vinelandii* CA11.1) had a

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faster electrophoretical mobility (Fig. 1, lanes 2 and 4) than VnfX from a mutant strain incapable of NifB-co production (*A. vinelandii* CA117.3(D*nifDK*D*nifB*), Fig. 1, lane 6), that had no V or Fe associated (as estimated by phosphorimaging and Fe staining of anoxic native gels, not shown). A portion VnfX purified from strain CA11.1 migrated at the higher position (Fig. 1, lane 4) probably due to dissociation of the V-Fe cluster from VnfX during purification. Treatment of V-deficient VnfX (purified from strain CA117.3) with purified NifB-co caused a shift in the migration of VnfX in anoxic native gels (Fig. 1, lane 7) to the same position of VnfX that has the V-Fe cluster bound to it (e.g. VnfX purified from strain CA11.1) (Fig.l, lane 2). The binding of NifB-co to VnfX was confirmed by incubating the partially purified protein with 55Fe-NifB-co. The 55Fe label was found associated with VnfX (Fig. 1, lane 3). Similarly, NifX can bind NifB-co, which changes its elctrophoretical mobility. We have also shown that NifX is required for *in vitro* FeMo-co synthesis. In vitro synthesis of FeMo-co with purified components (apodinitrogenase, NifNE, NifH and NifB-co) failed to generate significant FeMo-co, suggesting the requirement of one or more other components. Addition of purified NifX stimulated the synthesis of FeMo-co threefold (Shah et al. 1999). VnfX was unable to stimulate *in vitro* FeMo-co synthesis.

We have identified and characterized what appears to be a precursor of FeV-co that contains the heterometal (V). This intermediate is associated with VnfX, which suggests that this protein is involved in FeV-co synthesis. The step occurring on VnfX could be the addition of V to the partially formed cluster, since both a V-containing cluster and NifB-co, its Fe-S precursor can accumulate on VnfX.

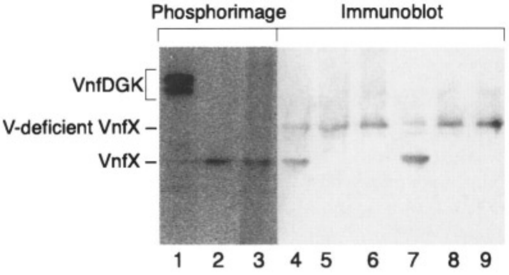

Fig. 1. Analysis of VnfX by anoxic, native gel electrophoresis. Phosphorimage and immunoblot developed with antibody to VnfX of an anoxic native gel. Lanes: 1. Extract of *A. vinelandii* CA12 (D*niJHDK*) grown in ^{49}V. 2 and 4. VnfX purified from *A. vinelandii* CA 11.1 (D*nifHDK*D*vnfDGK*) grown in ^{49}V. 3 and 7. VnfX purified from *A. vinelandii* CA117.3 (D*nifDK*D*nifB*) grown in V incubated with ^{55}Fe-NifB-co. 5. VnfX purified from *A. vinelandii* CA11.1 exposed to air for 5 min before electrophoresis. 6. VnfX purified from *A. vinelandii* CA117.3 . 8. VnfX purified from *A. vinelandii* CA117.3 incubated with air-inactivated NifB-co. 9. VnfX purified from *A. vinelandii* CA117.3 incubated with SB12 (final conc.0.03%) in 0.025 M Tris HC1 pH 7.4.

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STUDIES ON THE MECHANISM FOR THE ACTIVATION OF IRON AND SULFUR FOR FORMATION OF THE NITOGENASE METAL CENTERS

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The *A. vinelandii nifU* and *nifS* gene products (NifU and NifS) are proposed to have specific roles in the formation or repair of the [Fe-S] cores of metalloclusters contained within the catalytic components of nitrogenase. NifS is a pyridoxal phosphate-dependent L-cysteine desulfurase that is able to catalyze the *in vitro* reconstitution of an apo-form of the nitrogenase Fe protein whose [4Fe-4S] cluster has been removed by chelation. Although a specific function for NifU in nitrogenase [Fe-S] cluster formation is not known, the available evidence points to a role either as the iron source necessary for [Fe-S] cluster formation or as an intermediate site for [Fe-S] cluster assembly. For NifU to serve either of these functions it must have the ability to transiently bind iron destined for [Fe-S] cluster formation. Previous work has shown that isolated NifU is a homodimer that contains two identical [2Fe-2S] clusters. It seems unlikely, however, that these [2Fe-2S] clusters represent the source of iron necessary for nitrogenase [Fe-S] cluster formation, since they are tightly bound within the NifU protein and cannot be removed even with strong chelation reagents. Thus, we have speculated that the redox-active $[2Fe\text{-}2S]^{2+,+}$ clusters contained within NifU (E_m = -254 mV *vs* SHE), have a redox role involved in the binding or release of Fe or an [Fe-S] cluster intermediate at a second site within NifU. The NifU protein contains nine cysteine residues, Cys^{35}, Cys^{62}, Cys^{106}, Cys^{137}, Cys^{139}, Cys^{172}, Cys^{175}, Cys^{272} and Cys^{275}. Based on primary sequence comparisons to other [2Fe-2S]-containing proteins, NifU residues Cys^{137}, Cys^{139}, Cys^{172} and Cys^{175} are the most likely cluster-coordinating residues. Thus, any or all of the remaining cysteine residues could be involved in binding iron at a second site or participate in the assembly of an [Fe-S] cluster intermediate.

Comparison of NifU primary sequences to other proteins contained within the data base has led to speculation that NifU is a modular protein organized into distinct structural domains . One of these modules includes the central portion of NifU which encompasses the proposed [2Fe-2S] cluster ligands. Another module corresponds to the N-terminal third of NifU and it contains residues Cys^{35}, Cys^{62}, Cys^{106} . Counterparts to each of the cysteine residues contained within the proposed N-terminal module of NifU are also strictly conserved in a family of small proteins designated IscU. In the cases of *Escherichia coli, Azotobacter vinelandii,* and a variety of other prokaryotic organisms, the *iscU* gene is cotranscribed with another gene designated *iscS* . The IscS protein shares considerable primary sequence identity when compared to NifS and it exhibits the same L-cysteine desulfurase activity as NifS. Homologs to *iscU* and *iscS* are also widely conserved in nature. For example, a search of the protein database reveals that homologs to these genes are encoded within the yeast, mouse, arabidopsis and human genomes. Indeed, other workers have suggested that the N-terminal domain of NifU represents one of the most highly conserved protein sequence motifs in nature. The wide conservation of *iscS* and *iscU* genes in nature, and their apparent co-transcription in many prokaryotic organisms, indicates that IscU and IscS might have housekeeping roles involved in the general mobilization of Fe and S for [Fe-S] cluster formation. If

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this is correct, the cysteine residues conserved between NifU and the IscU family of proteins are most likely to be involved in providing the proposed second iron-binding site within NifU.

The functional significance of each of the nine cysteine residues encoded within the *A. vinelandii nifU* gene was assessed by site-directed mutagenesis and gene-replacement techniques. Purification and spectroscopic characterization of NifU proteins altered in this way were also used to identify the [2Fe-2S] cluster-coordinating cysteine residues. The existence of a labile rubredoxin-like Fe(III)-binding site in NifU was demonstrated by a combination of optical absorption and resonance Raman spectroscopies. The modular nature of the NifU polypeptide and the ligands to both metal sites were also addressed by spectroscopic and redox studies of the purified N-terminal and C-terminal fragments of NifU. These fragments were shown to contain the labile monomeric Fe-binding site and the indigenous [2Fe-2S] cluster, respectively, each with properties very similar to those established in holo NifU.

A second series of experiments was performed in order to determine if NifU is able to provide an intermediate site for [Fe-S] cluster formation. In this work it was shown that NifU is able to interact with NifS and, in the presence of ferric ion, L-cysteine, and catalytic amounts of NifS, a second "transient" [2Fe-2S] cluster type can be assembled in NifU *in vitro,* with a stoichiometry of approximately one cluster per homodimer. The transient [2Fe-2S] cluster species is labile and rapidly released upon reduction. It is therefore proposed that transient [2Fe-2S] cluster units are formed on NifU and then released to supply the inorganic iron and sulfur necessary for maturation of the nitrogenase component proteins. As mentioned above, the role of the permanent [2Fe-2S] clusters contained within NifU is not yet known but they could have a redox function involving either the formation or release of labile [2Fe-2S] cluster units assembled on NifU.

STOPPED-FLOW INFRA-RED SPECTROSCOPY OF CARBON MONOXIDE BINDING TO FUNCTIONING NITROGENASE

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The IR spectrum of the nitrogenase-CO complex depends on CO concentration (George et al. 1997, Thorneley and George) Under stoichiometric FeMo-cofactor:CO conditions ("low-CO") a single v(CO) stretch at 1904 cm^{-1} is seen, whose time-course shows a maximum intensity at 7 seconds (Figure 1). After 7 seconds, this 1904 cm^{-1} band decays to form a novel species with v(CO) at 1715 cm^{-1}. In the presence of excess (10-fold) CO ("high-CO"), a much more complex pattern of bands is seen. The maximum intensity of the 1904 cm^{-1} band is reduced with additional peaks slowly growing at 1958 cm^{-1}, 1936 cm^{-1} and 1880 cm^{-1}. The increase in intensities of the 1936 cm^{-1} and 1880 cm^{-1} bands with increasing CO concentration directly correlates with the loss of intensity of the 1904 cm^{-1} band. The apparent shift of the "low-CO" 1904 cm^{-1} to 1906 cm^{-1} apparently arises from the growth of a high-CO band at ~1908 cm^{-1}. That all these bands arise from bound CO was confirmed by use of ^{13}CO which caused the spectrum to shift ~ -44 cm^{-1}. As these bands decay the species with v(CO) at 1715 cm^{-1} is still formed but at a significantly lower rate.

SF-FTIR yields complementary information to EPR spectroscopy and other techniques. We find that the variation of the "high-CO" and "low-CO" EPR signals with time and CO concentration is qualitatively similar to, but does not directly correlate with, the FTIR data. We are interpreting this in terms of EPR being sensitive to specific paramagnetic redox states whereas FTIR observes all bound CO and, in a cluster, may be relatively insensitive to cluster oxidation state.

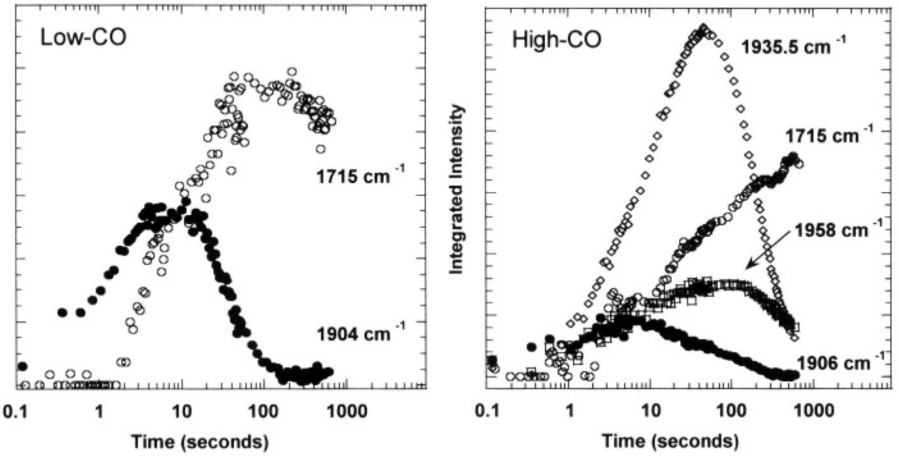

Figure 1 Time dependence of the principle bands observed in the *Klebsiella* nitrogenase-CO complexes.

The kinetics of inhibition have been probed using SF-FTIR monitoring the consumption of an alternative substrate, azide, which has a characteristic IR absorption at 2048 cm^{-1}. We find that the formation of the low-CO 1904 cm^{-1} band is concomitant with the onset of substrate inhibition.

The IR spectra and time-courses of the high-CO spectrum yield structural information about CO binding. Interestingly, the time courses of the 1936 cm^{-1} and 1880 cm^{-1} are identical, while those at

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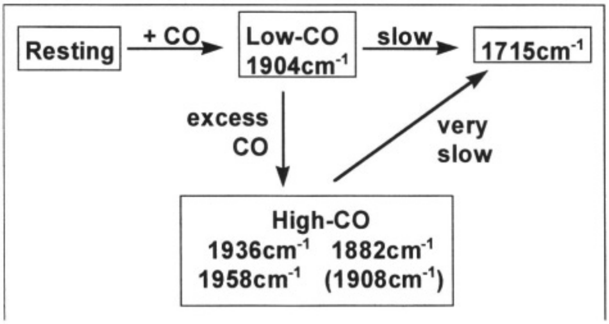
1904 cm^{-1} and 1958 cm^{-1} are distinct. In addition, spectra generated using a mixture of CO isotopes; $^{12}C^{16}O$ and $^{13}C^{18}O$ show a complex pattern of bands that are not a simple sum of the pure $^{12}C^{16}O$ and $^{13}C^{18}O$ spectra. Our interpretation of these data is that the 1936 cm^{-1} and 1880 cm^{-1} are the asymmetric and symmetric coupled vibrations of two CO molecules bound to the same metal site. If this two COs are equivalent then the strong intensity of the 1936 cm^{-1} compared to the 1880 cm^{-1} absorption suggests these two COs are *trans* to each other, with a OC-M-CO angle calculated to be 130 - 150°. The 1958 cm^{-1} and 1904 cm^{-1} bands do not appear to be coupled and so arise from isolated M-C≡O species.

The energies of these bands are sensitive to mutagenic replacement of amino-acid residues adjacent to the FeMo-cofactor. While wild-type *Azotobacter vinelandii* nitrogenase yields essentially identical spectra, the His-195-Gin variant gives significantly different v(CO) energies under both low (1913 cm^{-1}) and high-CO (1934 cm^{-1}, 1905 cm^{-1}) conditions. By contrast, use of $NifV^-$ nitrogenase, where the homocitrate on FeMo-cofactor is replaced by citrate, does not appear to affect the form of these spectra. Similarly the energies of the all the bands are unchanged in deuterated buffer, implying that bound CO is not hydrogen bonded to exchangeable protons.

Our model for the CO inhibition chemistry is illustrated in scheme 1. Stoichiometric CO binds and inhibits dinitrogen reduction. This is probably a M-C≡O terminal complex. This species slowly converts to the 1715 cm^{-1} form. In the presence of excess CO, additional, adventitious binding occurs at more than one metal site, including a second CO binding at the same metal atom as the 1904 cm^{-1} carbonyl. This slows the formation of the final 1715 cm^{-1} species, which forms as the adventitious CO molecules dissociate. The nature of the long lived 1715 cm^{-1} species is intriguing. An attractive possibility is that CO has become bound doubly or triply bridged to one face of the FeMo cofactor. Understanding the chemical nature of this species will provide insights to the reactivity of the nitrogenase FeMo-cofactor. We are currently attempting to crystallize this material, which would give us the first defined structure of a small molecule bound to nitrogenase.



Scheme 1 Interconversions of CO bound forms of *Klebsiella pneumoniae* MoFe protein during turnover in the presence of dinitrogen .

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REDUCTANT-DEPENDANT ATP UTILIZATION DURING NITROGENASE CATALYSIS: STUDIES USING TI(III)

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1. Introduction

Nitrogenase is a complex enzyme system comprised of two dissimilar metalloprotein components: the MoFe protein ($\alpha_2\beta_2$, M_r = 230,000 da) and the Fe protein (α_2, M_r = 63000 da). During catalysis, both proteins react with each other and electrons transferred from the Fe protein to the MoFe protein with concomitant ATP hydrolysis (Burgess, Lowe, 1996). After sufficient electrons have been transferred to the MoFe protein, substrate reduction occurs. Most studies use dithionite (DT) as reductant during which the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1-}$ redox couple of the Fe protein functions in the electron transfer role. However, the characterization (Watt, Reddy, 1994; Angove et al., 1998) of the $[Fe_4S_4]^0$ redox state of the Fe protein cluster suggests that other reactivity patterns may be observed using the Fe protein $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple. We have examined this possibility using Ti(III) known to form the $[Fe_4S_4]^0$ cluster state of the Fe protein and report that the ATP utilization requirement is decreased from 4.0-5.0 using DT to 2.0 using Ti(III).

2. Procedures

Ti(III) solutions were prepared as previously described (Seefeldt, Ensign, 1994). Nitrogenase proteins were obtained from Azotobacter vinelandii (Av) and Clostridium pasteuranum (Cp). Nucleotides were determined by HPLC and product formation was determined by previously established gas chromatography and ammonia methods (Burgess et al., 1980).

3. Results and Discussion

The amount of MgATP hydrolyzed per pair of electrons transferred (ATP/2e) during nitrogenase catalysis (1.0 atm N_2, 10% acetylene in argon or 100% argon, at 30 °C) using Ti(III) citrate as reductant was measured and compared to the same reaction using DT. ATP/2e values near 2.0 for Ti(III) and 5.0 for DT indicate that nitrogenase has a much lower ATP requirement using Ti(III) as reductant. Using reduced Av flavoprotein ($AvFLPH_2$), ATP/2e values near 2.0 were also observed. With Ti(III) as reductant ATP/2e values were independent of the substrate being reduced. An ATP/2e value of 2.0 was also observed in the presence of 5% CO. ATP/2e values measured as a function of temperature using DT and Ti(III) and for DT the ATP/2e values increased with increasing temperature but remained constant at 2.0 using Ti(III) as reductant. These latter results suggested that at least two reactions are occurring with DT but only a single reaction occurs with Ti(III). Both $AvFLPH_2$ and Ti(III) are capable of forming the $[Fe_4S_4]^0$ cluster state of the Fe protein whereas DT only forms the $[Fe_4S_4]^{1+}$. These results suggest that ATP/2e values arise from operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple with only 2 ATPs hydrolyzed per pair of electrons transferred. Additional experiments showed that ATP/2e values near 2.0 correlated with slower rates of product formation and that faster rates of product formation produced ATP/2e values near 5.0. ATP/2e values of 5.0 are consistent with operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$redox couple while ATP/2e values of 2.0 could arise from the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple. To understand this change in ATP utilization with variation of conditions with Ti(III) as reductant, we examined

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the rate of reduction of $[Fe_4S_4]^{2+}$ and $[Fe_4S_4]^0$ by Ti(III). Both $[Fe_4S_4]^{2+}$ and $[Fe_4S_4]^0$ are reduced at the same rate suggesting that Ti(III) reduction of $[Fe_4S_4]^{1+}$ to $[Fe_4S_4]^0$ is the slow step. This rate is comparable to that of nitrogenase turnover and controls whether $[Fe_4S_4]^{1+}$ or $[Fe_4S_4]^0$ is the dominant Fe protein species during nitrogenase catalysis. Slow turnover conditions favor formation of $[Fe_4S_4]^0$ that gives rise to ATP/2e values of 2.0 while rapid turnover conditions favor formation of $[Fe_4S_4]^{1+}$ that leads to ATP/2e values of 5.0. These results suggest that two distinct Fe protein redox couples may be functional during nitrogenase using Ti(III) and that the efficiency of ATP utilization depends upon which of these redox couples is dominant.

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5. Acknowledgements

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COFACTOR REACTIVITY AND MODELS FOR COFACTOR REACTIONS.

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This session extended the discussion of the biochemistry of nitrogenase proteins and cofactors (Parallel Session 2) to the chemistry of isolated cofactor and model systems aimed at understanding cofactor function.

1. Studies of isolated FeMoco.

Isolation of FeMoco from the MoFe protein of *A. vinelandii* and *K. pneumoniae* is now sufficiently developed to provide quantities that allow study of substrate interaction and reduction by electrochemical and chemical techniques.

Thin-layer FTIR spectroelectrochemical studies of FeMoco by Pickett et al. showed that CO binds terminally to reduced FeMoco (in the absence or presence of PhSH and imidazole), giving a range of v(CO) values close to those observed during the turnover of nitrogenase under CO. This has now been extended to the interaction of Bu^tNC, which gives rise to two NC stretching bands for terminally bound Bu^tNC at oxidised, epr-silent FeMoco, which shift as expected to lower values upon reduction to the semi-reduced state of the cofactor. Although it appears that neither CO nor Bu^tNC bind to the terminal Fe, the sites at which CO and Bu^tNC bind are not established and model studies using metal complexes of these ligands, such as those discussed below, are providing information to aid in deciding upon the likely sites of ligand binding.

Shilov discussed catalytic reduction of N_2 in protic media, highlighting the coupled electron transfer from the external agent (P-cluster) to FeMoco containing bound N_2, followed by electron transfer within this cluster. Experimental investigation of the latter type of process for FeMoco has been studied by Bazhenova et al. who have shown that in nmf or thf solution, FeMoco catalyses the conversion of C_2H_2 to C_2H_4 and traces of C_2H_6 when reduced with Zn/PhSH or Eu/Hg/PhSH. From kinetic studies there appear to be multiple sites for reduction accessed by the more strongly reducing Eu/Hg system, and H_2 is also evolved. The reduction is inhibited by CO and more importantly, by N_2. Future work is planned to establish that FeMoco retains its integrity throughout these reactions.

2. Model studies

Major advances in the chemistry of N_2 were reported by Hidai. He has shown that certain acidic H_2 complexes are capable of protonating N_2 in classical tungsten N_2 complexes. Though this kind of reactivity is not entirely novel, the fact that the H_2 complex can be generated *in situ* from H_2 means that the protonation reaction is effectively one between N_2 and H_2, yielding NH_3. It is thus a formal analogue of the Haber process but is carried out at 55 °C and 1 atm H_2.

In addition, Hidai has shown that bridging hydrosulfido-groups can react with coordinated N_2 to yield some NH_3, though the mechanism and a satisfactory rationale are still lacking. The reactions demonstrate for the first time that hydrosulfide protons can react with N_2, a mechanism that has been proposed on more than one occasion to be involved in nitrogenase activity.

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Richards et al. described a series of researches aimed at obtaining N_2 coordinated to V, Mo or Fe in a sulfur environment, such as apparently exists in the nitrogenase cofactor clusters. Although they succeeded in preparing such a complex for vanadium, this proved to be very unstable. However, they did succeed in isolating a wide range of products that can be regarded as analogues of N_2-protonation intermediates or of nitrogenase-substrate complexes. Some Fe carbonyl complexes were described which show v(CO) values in the same range as those typical of FeMoco. Richards also described some EHMO calculations on the interaction of N_2 with FeMoco that point to N_2 binding to Mo and then splitting to form nitrides on both Mo and Fe. Experimental support for this proposal is being sought.

Finally, Leigh et al. described a series of model studies that show: (a) that the preferred mode of binding of N_2 to V^{II} or V^{III} is bridging between two vanadium atoms, which can be ruled out in nitrogenases, though a hetero-bridge, $V\text{-}N_2\text{-}Fe$, may be possible; (b) that the reduction of cyclopropenes in model systems involves a cyclopropyl intermediate that either is protonated (to the cyclopropane) or opens (to the propene) and that this implies limited access of protons to the cyclopropene-binding site of nitrogenases; and (c) that the mechanisms of substitution reactions of metal trihydro-complexes are dissociative rather than associative, which militates against a single metal trihydride being the active site in nitrogenases, and which implies that the hydrides, if involved at all, are distributed over more than one metal site.

INTERACTIONS OF SMALL MOLECULES WITH ISOLATED FEMOCO

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Direct studies of isolated FeMoco have certain advantages over studies on the whole enzyme system, notably: the possibility of selectively accessing redox-states; the exclusion of complications which might arise from the presence of the other metallo-sites within the proteins; the opportunity to widely and systematically control both the co-ligand and the outer-sphere environment of the catalytic centre.

A combination of electrochemical and spectroelectrochemical methods have shown for the first time that the π-acid ligand CO binds to isolated FeMoco when it is reduced from the epr active semi-reduced state (Legall et al 1999). In addition it has been shown that FeMoco is capable of catalysing the reduction of protons at high potential (Ibrahim et al 1999). We are thus beginning to see that the extracted cofactor can engage in electron transfer chemistry which has some of the attributes of the whole enzyme system.

We have now turned our attention to the interaction of alkyl isocyanides with FeMoco for the following reasons. First, coordinated RNC is IR active and should provide a sensitive probe in spectroelectrochemical studies. Secondly, it should bind to cofactor sites where CO binds, but possibly in higher oxidation states, and finally it is a potential substrate.

We find that tBuNC interacts with FeMoco when the cofactor is in its oxidised and semi-reduced forms. FTIR spectroelectrochemical studies have allowed the detection of bands attributable to the bound ligand in the oxidised state at 2166 and 2136 cm^{-1} which shift to 2146 and 2099 cm^{-1} upon one electron reduction. The shift upon reduction of these terminal stretches to a lower frequency is as expected for π-acid ligands.

Taking this data together with information from competitive studies with thiolate and CO strongly suggests that at least one isocyanide ligand binds to a core iron atom and that probably a second ligand binds at a Mo site which is occupied by histidine when the cofactor is protein bound.

The figure shows the FTIR thin-layer spectroelectrochemical data obtained for reduction and re-oxidation of the oxidised form of the isocyanide modified cofactor. A curve fitting analysis of this difference spectrum provides the absorption frequency data for the terminal bound isocyanide ligands.

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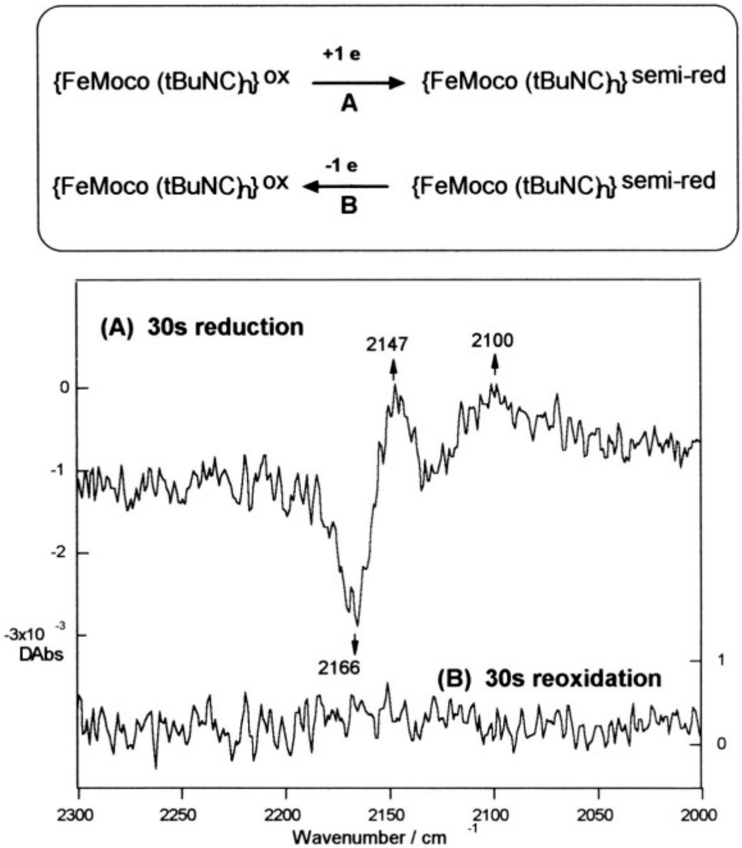

Figure : FTIR Difference Spectra. (A) reduction of tBuNC modified FeMoco (ox). (B) re-oxidation of semi-reduced state.

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Acknowledments

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CATALYTIC REACTIONS WITH FeMoco IN NON-ENZYMATIC SURROUNDINGS (COMPARISON WITH SYNTHETIC CATALYSTS)

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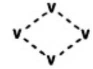
1. Introduction

FeMo cofactor of nitrogenase, the cluster of the composition Fe_7MoS_9 (homocitrate), has been shown to catalyze the reduction of acetylene and some other nitrogenase substrates and also to coordinate dinitrogen when bound to the surface of a multi-electron donor: Zn, Eu and Na amalgams (see T.A. Bazhenova et al, these Proceedings). Using electrochemical terminology P cluster of nitrogenase may be considered "nano-electrode" to which FeMoco is attached. To find the conditions for FeMoco functioning in non-enzymatic surroundings we used the same electron donors as with synthetic catalysts active with N_2 found some time ago. Now for the first time we can compare on the one hand catalytic functions of the FeMoco in natural and non-protein surroundings and also on the other hand FeMoco and synthetic complexes active with dinitrogen and other nitrogenase substrates when attached to the surface of the same electron donors at least for acetylene.

2. Stoichiometric N_2 reduction in protic media

Similarly to the enzymatic process dinitrogen reduction in protic media involves electrons from the reducing agent and protons ultimately from the media. All the systems known so far reducing dinitrogen in protic media are polynuclear (A.E. Shilov, 1997). Presumably polynuclear structures with the possibility of N_2 coordination by at least four metal atoms helps to avoid two-electron reduction of N_2 producing thermodynamically unfavorable diazene, N_2H_2. Among known complexes reducing dinitrogen stoichiometrically the complexes of V^{2+} with catechols form a unique family of homogeneous water and alcohol solutions in which N_2 is reduced to form $2NH_3$ and H_2 i.e. with the stoichiometry similar to that of enzymatic N_2 reduction with Mo nitrogenase:

$$8V^{2+} + N_2 + 8H_2O \rightarrow 8V^{3+} + 2NH_3 + H_2 + 8OH^-$$

X-ray analysis for the system V^{2+}-3,5-di-*tert*-butylcatechol reveals the formation of four-nuclear species with  a core.

Recent kinetic results show that two such species interact to reduce N_2 to ammonia, one apparently with coordinated dinitrogen, the other providing two additional electrons. When acid is added to N_2 reducing solutions of V^{II}-catechol complexes hydrazine is formed in a four-electron reaction of one four-nuclear species. Thus water is a sufficiently strong acid to produce ammonia in an octa-nuclear intermediate, but to form hydrazine from the four-nuclear complex a stronger acid is needed.

3. Catalytic N_2 reduction

Polynuclear molybdenum(III) complexes were found to catalytically reduce dinitrogen to hydrazine and ammonia when bound to semiconducting $MgTi_2O_4$, sodium or europium amalgams or $MgTi_2O_4$ + mercury cathode (A.E. Shilov, 1997). An octanuclear complex was isolated which (when bound to multi-electron donor) is an active catalyst for N_2 reduction at room temperature and atmospheric

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pressure. In the stoichiometric reaction the oxidation state of the transition metal increases in the process of N_2 reduction by one unit each. For the catalytic reaction one obvious mechanism could include the similar independent process of the cluster followed by the stepwise reduction of the oxidized cluster by the external reductant to regenerate the catalytically active species. However the mechanism for the catalytic reduction of dinitrogen is distinctly different. Thus when the catalytic complex is fully reduced (in the case of molybdenum cluster to Mo^{III} state) no noticeable (even stoichiometric) reduction of N_2 takes place in the absence of the reductant. Therefore, we conclude that the external electron donor takes part in other steps of the catalytic cycle, not only in the regeneration of the catalyst. This participation may be envisaged as an additional electron transfer from the external donor after the coordination of the substrate and subsequently in the rate-determining step of the substrate transformation into the hydrazine derivative involving also the protonation of dinitrogen. Indeed after the coordination of N_2 the electron capacity of the whole system will be increased as well as positive charges on metal atoms. The subsequent transformation induced by the protonation of coordinated dinitrogen will again increase positive charges on metal atoms. The crucial step of irreversible transformation of the substrate will be facilitated by one-electron transfer from the electron donor ("reductive coaction"), therefore, coupled one-electron transfer from the external donor and multi-electron transformation of the substrate in coordination sphere of the complex will be mutually beneficial.

In agreement with this mechanism the reaction rate with both synthetic catalysts and isolated FeMoco depends on the redox potential of the reductant (according to the Tafel equation). This mechanism also explains the role of the P cluster in nitrogenase functioning: electron transfer from the P cluster takes place as a coupled process with dinitrogen reduction in the coordination sphere of FeMoco, apparently facilitating substrate reduction. This was the idea behind our attempts to use FeMoco as the catalyst in non-biologic surroundings. To be catalytically active FeMoco has to be bound to a multi-electron donor (such as amalgams or cathode) and to be open to an acid with a certain pK which ensures the synchronous protonation and electron transfer. It seems that we have solved the first part of the problem and have shown that dinitrogen is able to coordinate to the isolated FeMoco. The second part is to find a suitable proton donor.

At present the following general features of the catalysis with FeMoco and synthetic catalysts could be noted:

a. Several, often eight metal atoms participate in the process with synthetic clusters and FeMoco. V^{II} and Mo^{III} are stronger reductants than Fe^{II} in FeMoco, but sulfur bridges (instead of oxygen bridges in the model systems) increase the reducing power of FeMoco.
b. Acetylene is readily reduced to ethylene or to ethylene + ethane (depending on electron flow). CO strongly inhibits the reaction. N_2 is coordinated and reduced in the case of the synthetic catalysts, while inhibiting acetylene reduction in the case of FeMoco.
c. The mechanism of catalysis involves "reductive coaction" of the external electron donor, i.e. coupled one-electron transfer to the catalytic complex and poly-electron reduction of a substrate. This coaction helps to transform even such "difficult" substrate as N_2 to a hydrazine derivative by not very strong reducing agents relatively stable in protic media.
d. Thiophenol is an essential co-catalyst at least for low-potential reductant (Zn amalgam) taking part not only in protonation but also in bridging the electron donor and the catalyst.

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CATALYTIC BEHAVIOR OF ISOLATED FeMo-COFACTOR OF NITROGENASE IN NON-PROTEIN SURROUNDINGS

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Our previous studies have established that the isolated FeMoco, the active site of the nitrogenase, can catalyze the reduction of acetylene and some other nitrogenase substrates in non-protein conditions, using Zn(Hg) or Eu(Hg) as reducing agent and thiophenol as a source of protons and an important cocatalyst (Bazhenova et al, 1997, 1998). To understand the mechanism of FeMoco catalytic activity in non-enzymatic conditions and to compare its catalytic behavior with that of the nitrogenase "M" center, we studied the kinetics of C_2H_2 reduction by Zn and Eu amalgams catalyzed by isolated FeMoco and inhibition of this process by CO. FeMoco was isolated from MoFe protein of *Azotobacter vinelandii* (McLean et al, 1989). Extracted FeMoco's specific activity, when reconstituted into Kp5058, was (200±20) nmol C_2H_4 produced min^{-1} $nmol^{-1}$ of Mo. Different FeMoco redox levels are reached in the presence of different reducing agents: Zn amalgam (-0.84V vs. NHE) (I) and Eu amalgam (- 1.4V vs. NHE) (II). Using (I) as a reductant, we observed a normal hyperbolic response in Michaelis-Menten plots and a K_m of 0.045 atm of C_2H_2. With (II), FeMoco-catalyzed C_2H_4 and C_2H_6 production as a function of the C_2H_2 pressure showed sigmoidal kinetics, which is consistent with substrate-induced cooperativity among 3 sites for C_2H_2 reduction. One site operates below 50 mm of C_2H_2 with a K_m of 0.006 atm of C_2H_2, which is the same for the formation of both C_2H_4 and C_2H_6. Between 50-220 mm of C_2H_2 at least two other sites are active (Hill coefficient of 1.6) with an apparent K_m for C_2H_2 of 0.08 atm (Fig.1). The specific activity was 3-5 nmol C_2H_4 min^{-1} $nmol^{-1}$ Mo for (I) and 40-60 nmol C_2H_4 min^{-1} $nmol^{-1}$ Mo for (II).

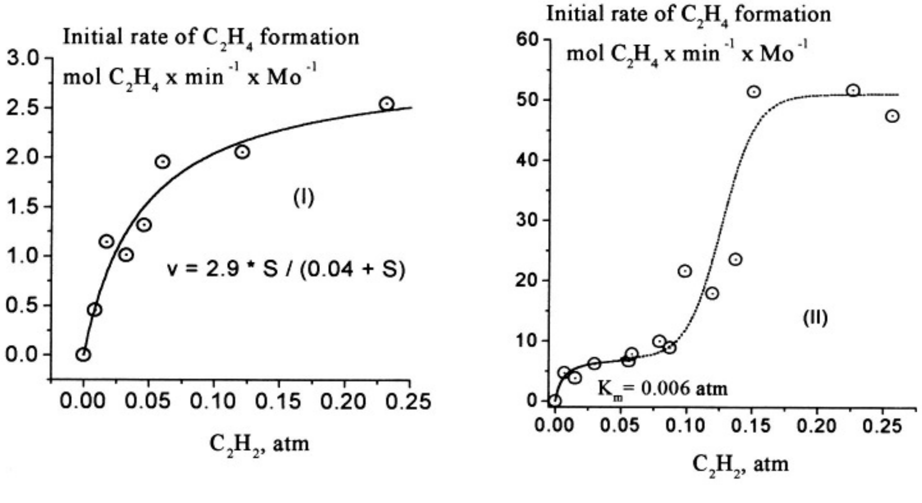

Figure 1. Michaelis-Menten plot of C_2H_2 reduction to C_2H_4 catalyzed by FeMoco in DMF with Zn(Hg) (I) or Eu(Hg) (II) as a reductant.

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The cofactor extracted from MoFe-proteins from *A. vinelandii* or *Klebsiella pneumoniae,* reveal the same catalytic behavior under the identical conditions. Besides C_2H_2 reduction, FeMoco catalyzes H_2 evolution in both the (I) and (II) systems. Moreover, the stronger the reducing agent used, the higher rate of H_2 evolution observed.

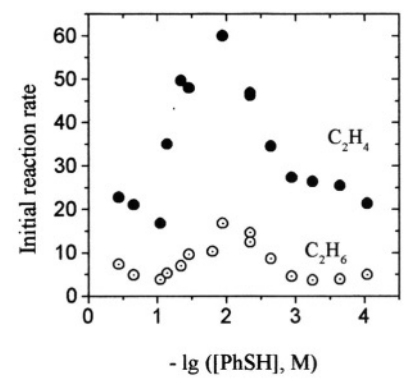

Fig. 2.
Dependence of initial reaction rate on thiophenol concentration

Fig. 2 shows the dependence of the rate of acetylene reduction to C_2H_4 and C_2H_6 by system (II) on the thiophenol concentration. A similar bell-shaped profile was observed for the dependence of C_2H_2 reduction by nitrogenase *in vitro* on pH (Pham, Burgess, 1993). Such a dependence of reaction rate on acid concentration shows a complicated protonation mechanism of the coordinated substrate (Henderson, 1996).

CO acts as an inhibitor of acetylene reduction. The K_i of CO varies with the potential of the reducing agent, indicating that different FeMoco redox levels interact with CO differently. For (I), CO inhibition of C_2H_2 reduction is reversible with a K_i = 0.05 atm. For (II), the inhibition constants for ethylene and ethane formation are different, K_i (atm) = 0.004 for C_2H_4 and 0.009 for C_2H_6. Distinct C_2H_2-binding sites are inhibited by CO differently. For (II), C_2H_2 reduction is very sensitive to N_2 inhibition as well (Fig. 3), indicating that N_2 binds to isolated and reduced FeMoco in non-protein conditions. These results allow us to conclude that FeMoco shows a high level of self-sufficiency and can realize most of its functions without the protein; it is only necessary to provide the conditions for the transfer of electrons and protons to the activated substrate molecule.

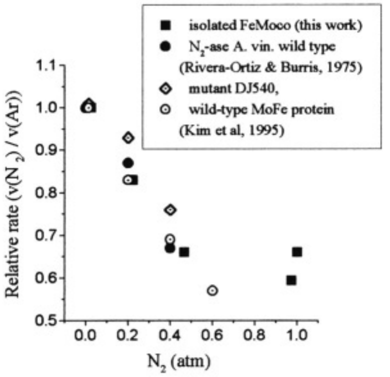

Fig. 3.
N_2 inhibition of C_2H_2 reduction for different protein and non-protein systems with FeMoco as a catalyst.

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CHEMICAL NITROGEN FIXATION: PROTONATION OF COORDINATED DINITROGEN WITH COORDINATED DIHYDROGEN OR BRIDGING HYDROSULFIDO LIGANDS

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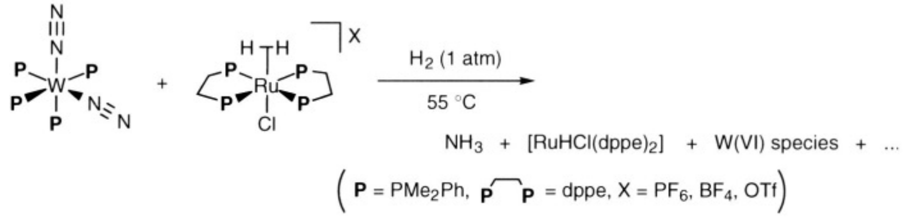
Industrial nitrogen fixation from dinitrogen(N_2) and dihydrogen(H_2) has been carried out for more than 80 years by the use of Fe-based heterogeneous catalysts, but the reaction conditions are extremely severe. In contrast, biological nitrogen fixation occurs at ambient temperature and atmospheric pressure. Thus, one of the most challenging subjects in chemistry is development of a new chemical nitrogen fixation which proceeds under mild conditions with the aid of specially designed catalysts. Here we describe our recent study toward this direction.

Synthesis of ammonia by reactions of tungsten dinitrogen and ruthenium dihydrogen complexes under mild conditions

We have long been interested in the reactivities of the coordinated N_2 in complexes of the type $[M(N_2)_2(L)_4]$ (M = Mo, W; L = tertiaryphosphine) because of their possible relevance to biological nitrogen fixation and the rich chemistry of the coordinated N_2 (M. Hidai, Y. Mizobe, 1995). Although the ligating N_2 was transformed into ammonia by treatment with inorganic acids such as H_2SO_4 (J. Chatt et al., 1975), H_2 could not be used for the N-H bond formation. We have recently found the ruthenium-assisted protonation of coordinated N_2 on tungsten with H_2 (Y. Nishibayashi et al., 1998). Treatment of *cis*-$[W(N_2)_2(PMe_2Ph)_4]$ with an equilibrium mixture of trans-$[RuCl(\eta^2\text{-}H_2)(dppp)_2]X$ with pKa = 4.4 and $[RuCl(dppp)_2]X$ [X = PF_6, BF_4, OTf (Tf = SO_2CF_3); dppp = 1, 3-bis(diphenylphosphino)propane] containing 10 equiv of the Ru atom based on the W atom in benzene-dichloromethane at 55 °C for 24 h under 1 atm of H_2 produced ammonia in 45~55% yields based on the W atom, together with the formation of *trans*-$[RuHCl(dppp)_2]$. Detailed studies on the reaction of *cis*-$[W(N_2)_2(PMe_2Ph)_4]$ with various $Ru(\eta^2\text{-}H_2)$ complexes revealed that the yield of ammonia produced critically depended upon the pKa value of the employed $Ru(\eta^2\text{-}H_2)$ complexes. When cis-$[W(N_2)_2(PMe_2Ph)_4]$ was treated with 10 equiv of trans-$[RuCl(\eta^2\text{-}H_2)(dppe)_2]X$ with pKa = 6.0 [X = PF_6, BF_4, OTf; dppe = 1, 2-bis(diphenylphosphino)ethane] under 1 atm of H_2, ammonia was formed in higher yields (up to 80%) compared with the above


N
N
P
P
W
P
P
N
N
P
H
H
X
P
Ru
P
P
Cl
+
H2 (1 atm)
55 °C
NH3 + [RuHCl(dppe)2] + W(VI) species + ...
P = PMe2Ph, P P = dppe, X = PF6, BF4, OTf

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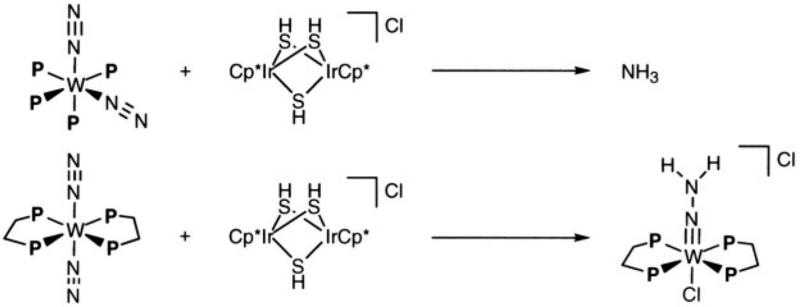
reaction. If the pKa value of a Ru(η^2- H_2) complex was increased to about 10, the yield of ammonia was remarkably decreased. In these reactions, heterolytic cleavage of H_2 seems to occur at the Ru center via nucleophilic attack of the coordinated N_2 on the coordinated H_2 where a proton (H^+) is used for the protonation of the coordinated N_2 and a hydride (H^-) remains at the Ru atom. Treatment of *cis*-[W(N_2)$_2$(PMe$_2$Ph)$_4$] or *trans*-[M(N_2)$_2$(dppe)$_2$] (M = Mo, W) with Ru(η^2-H_2) complexes at room temperature led to isolation of intermediate hydrazido(2-) complexes such as *trans*-[W(NNH$_2$)(X)(dppe)$_2$]$^+$ (X = OTf, F) and *trans*-[W(NNH$_2$)(OTf)(PMe$_2$Ph)$_4$]OTf. We presume that further ruthenium-assisted protonation of hydrazido(2-) intermediates such as *trans*-[W(NNH$_2$)(OTf)(PMe$_2$Ph)$_4$]OTf with H_2 at 55 °C results in the formation of ammonia along with W(VI) species. Our studies are now in progress toward development of bimetallic systems where both the hydrogen atoms of activated H_2 are effectively used for the catalytic nitrogen fixation from N_2 and H_2.

Protonation of coordinated dinitrogen with hydrosulfido-bridged dinuclear complexes

In biological nitrogen fixation, the bridging sulfido ligands in the FeMo-cofactor of nitrogenase is considered to mediate proton transfer to the activated N_2 bound to the Mo or Fe metal(s). Although treatment of N_2 complexes of the type *cis*-[M(N_2)$_2$(PMe$_2$Ph)$_4$] (M = Mo, W) with organic thiols or H_2S does not lead to the N-H bond formation, we have now found that the proton on the bridging sulfur in hydrosulfido-bridged dinuclear compounds of iridium and iron is transferred to the ligating N_2 on the W atom to form ammonia. The reaction of *cis*-[W(N_2)$_2$(PMe$_2$Ph)$_4$] with 10 equiv of [Cp*Ir(μ-SH)$_3$IrCp*]Cl (Cp* = η^5-C$_5$Me$_5$) or [P$_3$Fe(μ-SH)$_3$FeP$_3$]BF$_4$ [P$_3$ = bis(2-diphenylphosphinoethyl)phenylphosphine] in dichloroethane-benzene at 55 °C produced ammonia in moderate yield. When *trans*-[W(N_2)$_2$(dppe)$_2$] was employed, the hydrazido(2-) complexes such as *trans*-[WCl(NNH$_2$)(dppe)$_2$]Cl were isolated in high yields. Whether such proton transfer occurs in nitrogenase is still completely open to conjecture, however, this type of model system will provide valuable information about the mechanism of biological nitrogen fixation.



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MO, V AND FE COMPLEXES OF TRIPODAL SULFUR-DONOR LIGANDS AS MODELS FOR NITROGENASE ACTIVE SITES

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Nitrogen Fixation Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

The cofactor of Mo-nitrogenase (FeMoco) is the site at which dinitrogen is converted into ammonia. FeMoco contains an $MoFe_7S_9$ cluster with the Mo atom ligated by one nitrogen, three sulfur and two oxygen atoms. The V atom and the Fe atom in the alternative nitrogenases are probably in a similar environment (Howard, Rees, 1996). The chemistry of V, Mo and Fe centres which carry three sulfurs (plus other ligands) (MS_3 sites) is therefore of great importance in understanding the mode of action of these enzymes.

Dinitrogen can be bound and reduced to NH_3 at Mo, V or Fe phosphine-ligated centres, primarily at mononuclear metal sites, but N_2 in the bridging mode can also give ammonia. With the tripodal ligand $[N(CH_2CH_2NSiMe_3)_3]^{3-}$ (NN_3), the (μ-dinitrogen) complex, $[Fe\{N_2Mo(NN_3)\}_3]$, has been prepared along with the monomer $[Mo(NN_3)(N_2)]$ (see Richards, 1998). This chemistry and the S_3-ligand environment of metals in nitrogenase prompted us to explore the chemistry of V, Mo and Fe, carrying the tripodal ligand $N(CH_2CH_2S)_3^{3-}$ (NS_3), with respect to the binding of N_2, H_2, CN^-, CO and nitrogenous species, such as N_2H, N_2H_2 and N_2H_4. Our results are summarised in Figures 1 (for Fe), 2 (for Mo) and 3 (for V). Only for V was a compound containing N_2 observed, the unstable $\{(NS_3)V(\mu\text{-}N_2)V(NS_3)\}$, **A.** CO interacted only with Fe and no interaction was seen with H_2 for any metal. It is significant that only Fe has the ability to bind CO in the terminal mode, in the complex anion $[Fe(NS_3)(CO)]^-$ **B** and the linear Fe_3S_4 carbonyl cluster $[Fe\{Fe(NS_3)(CO)\text{-}S,S'\}_2]$ **C**. The IR C-O stretching frequencies of **B** (1885 cm^{-1}) and **C** (1937 cm^{-1}) are similar to IR frequencies observed in CO complexes of Klebsiella pneumoniae nitrogenase and FeMoco (1880, 1906, 1936 and 1958 cm^{-1}) (George et al., 1997; Ibrahim et al, 1999). The chemistry apparent for V and Mo with NS_3, in relatively high oxidation states, shows a tendency for these metals to form multiple bonds to nitrogenous ligands and to degrade hydrazine. This chemistry is echoed by that of cluster anions such as $[MoFe_3S_4Cl_3(C_6Cl_4O_2)(MeCN)]^{2-}$ and $[VFe_3S_4Cl_3(Me_2NCHO)_3]^-$ which do not interact with CO at Mo or V, but catalyse the disproportionation and reduction of hydrazine at these hetero-atoms (Coucouvanis, 1996).

These observations suggest that Fe is the site of CO binding to FeMoco in the terminal mode and Fe might also be the binding site of N_2. Theoretical studies favour binding of N_2 between two irons, either on an edge of the central cavity or across a face, perhaps cleaving to give nitrides as a first step (Richards, 1998). There are some model compounds to support such an interaction (Sellmann, 1996; Figure 1). Nevertheless, one cannot exclude as the site of N_2 activation the Mo atom, which could achieve N_2 binding by displacement of carboxylate, followed by interaction of bound N_2 with a neighbouring Fe to give Mo and Fe nitrides, which are then reduced to ammonia (M.C. Durrant, personal comm.). Nitrogenase action might involve initial binding of N_2 at the central Fe atoms of FeMoco, followed by partial reduction to hydrazine, which is then transferred to the Mo where the reduction to NH_3 is completed (Coucouvanis, 1996). Whilst this suggestion is in accord with much of the above chemistry, studies of sulfur-ligated Mo and V are needed before they can be excluded as the site of reduction of N_2. In particular, lower oxidation states of Mo and V need examination, in view of the existence of the Mo(0) complex anions $[Fe_3S_4L_3Mo(CO)_3]^{3-}$ (L=SEt, Cl) (Coucouvanis,

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1996) and the Mo(II) complex $[MoI_2(CO)_3\{Fe_4(C_5H_5)_4S_6\text{-}S'S''\}]$ (Baker et al, 1999), which also have CO stretching frequencies in the region noted above for CO derivatives of FeMoco.

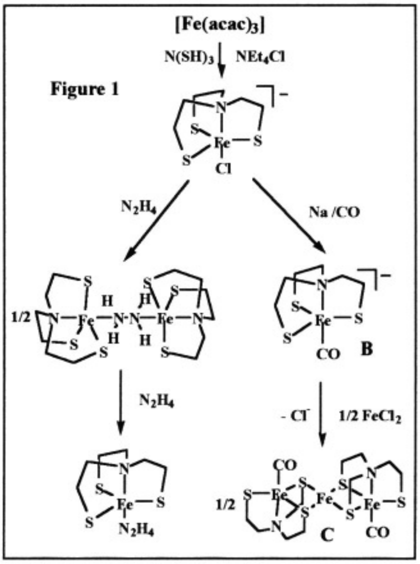
Figure 1
[Fe(acac)3]
N(SH)3
NEt4Cl
Cl
N2H4
Na /CO
1/2
B
CO
N2H4
- Cl-
1/2 FeCl2
N2H4
1/2
C
CO

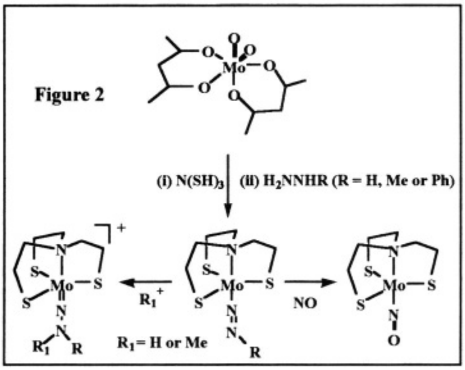
Figure 2
(i) N(SH)3
(ii) H2NNHR (R = H, Me or Ph)
R1+
R1 = H or Me
NO

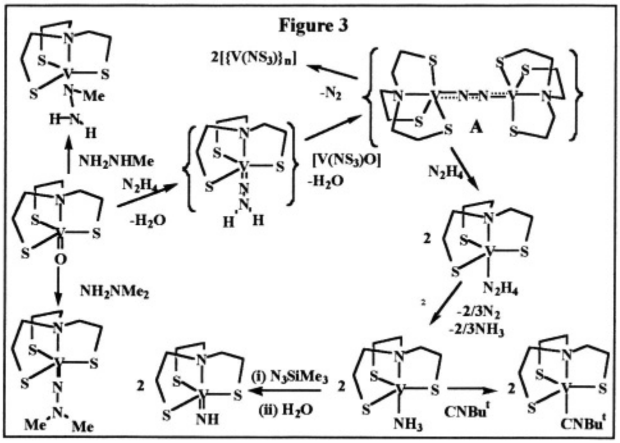
Figure 3
2[{V(NS3)}n]
-N2
A
NH2NHMe
N2H4
-H2O
[V(NS3)O]
-H2O
N2H4
2
N2H4
-2/3N2
-2/3NH3
NH2NMe2
2
(i) N3SiMe3
(ii) H2O
2
NH3
CNBut
2
CNBut

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THE USE OF CHEMICAL MODELS TO PROBE THE MECHANISMS OF SUBSTRATE REDUCTION REACTIONS OF NITROGENASES

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1. Introduction

It has now been shown by many examples that dinitrogen can bind to transition metal ions in a variety of ways (Leigh, 1992). In the largest single group the dinitrogen is bound at one end only, but it can bridge between two or more metal ions in various fashions. In some of these alkali metal ions are also involved, but there is little evidence that alkali metals alone can bind to dinitrogen. Generally the oxidation state of the transition metal is II or less. Few complexes with sulfur ligands bind dinitrogen.

The protonation of complexed dinitrogen to give ammonia and/or hydrazine has also been observed in several cases, generally in complexes of transition elements of Groups 4, 5, and 6 of the Periodic Table(Leigh, 1992). New dinitrogen complexes and their protonation will not necessarily add much to our understanding of the dinitrogen protonation process in nitrogenase. A more sophisticated use of models is needed, and we provide here three examples that demonstrate how this can be done.

2. Vanadium(II) dinitrogen complexes

Of vanadium dinitrogen complexes, those with vanadium(-I) contain singly bonded end-on dinitrogen and are isoelectronic with the well known phosphine complexes of molybdenum(0) and tungsten(0).They react with proton acids, ultimately to generate ammonia and/or hydrazine. However, such low oxidation states are unlikely to be attainable in biological systems. Those of vanadium(II) and vanadium(III) all contain dinitrogen bridging end-on between two vanadium ions. Examples include $[\{V(C_6H_4CH_2NMe_2)_2(C_5H_5N)\}_2(N_2)]$ (Edema et al. 1989) and $[\{V(CH_2Bu^t)_3\}_2(N_2)]$ (Buijink et al., 1993)

We have examined the interaction of a series of *N, C*-donors, such as $[Fe(C_5H_5)(C_5H_3CH_2NMe_2)]^-$, $C_6H_4CH_2CH_2NMe_2^-$ and $C_6H_4CH_2CH_2CH_2NMe_2^-$, with $[VCl_2(tmen)_2]$ (tmen = $Me_2NCH_2CH_2NMe_2$). Apparently only nitrogen and carbon donors allowed dinitrogen uptake, and the maximum uptake we ever observed was one dinitrogen per two vanadium ions. The products react with protic acids to give ammonia. It is likely that in every case these complexes, like those described in the literature, contain dinitrogen bridges.

Since there is only one vanadium atom in FeVaco, and since its oxidation state is unlikely to be lower than II, this argues against a single vanadium ion (or, by extension, molybdenum) being the whole active site at which dinitrogen binds. A more complicated binding involving vanadium/molybdenum plus iron is not excluded, but there are, as yet, few models of such binding, and no reported reactivity,

3. The mechanism of displacement of dinitrogen from metal complexes

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The Lowe-Thorneley mechanism (Lowe, Thorneley, 1984) of nitrogenase function requires three electrons to enter the larger nitrogenase protein before dihydrogen is displaced and dinitrogen is bound. This has been interpreted as indicating that a metal trihydride is generated (Leigh, 1995). Data for other substrates are generally lacking. The most widely accepted model of dinitrogen-mediated HD formation, like the Lowe-Thorneley model, seems to imply that the displacement of dihydrogen by dinitrogen is associative (Leigh, 1995).

$$[L_nMH_3] + N_2 \rightarrow [L_nMH_3(N_2)] \rightarrow [L_nMH(N_2)] + H_2$$

We have studied the kinetics of displacement of dihydrogen by dinitrogen, and the converse, in a series of Group 8 complexes such as $[MH(H_2)(dmpe)_2]^+$ (M = Fe, Ru, or Os) (Helleren et al., 1999). All these trihydrides react by a dissociative mechanism, and this is probably true for trihydrides in general.

$$[L_nMH_3] - H_2 \rightarrow [L_nMH] + N_2 \rightarrow [L_nMH(N_2)]$$

This observation undermines the associative model for HD formation and also shows that the simple trihydride interpretation of the Lowe-Thorneley kinetic model is inadequate. Our currently preferred interpretation is that if three hydrides are indeed generated, they are not all at the same site, and not necessarily at the site at which dinitrogen is bound (Leigh, 1995). The data argue strongly for a model in which dihydrogen evolution arises from a site other than that which binds dinitrogen. They also imply that CO might stimulate HD formation by binding at the dinitrogen site and stopping nitrogen fixation but not dihydrogen evolution.

4. The reduction of cyclopropenes by models and by nitrogenases

Cyclopropene itself is a substrate that gives rise to two products upon reduction by nitrogenases, namely propene and cycloopropane. These are both two-electron reduction products. The ratio of these products is generally 2:1, though other ratios have been reported (Gemoets et al., 1989). We have now studied the reduction of a range of cyclopropenes by various model systems, for example, after complexing them to platinum(0) and then treating them with protic acid (Leigh et al., 1998), and by direct reaction with iron polyhydrides (Hughes et al., 1999). The model systems reduce cyclopropene itself and cyclopropane is the principal product. However, the reaction products of cyclopropenes in general are of both cyclopropane and propene types, formed from a cyclopropyl complex intermediate. This ring-opens to give the propene product when the supply of protons form the solution is limited. We therefore infer that the environment of the cyclopropene-reducing site in nitrogenase (not necessarily the dinitrogen-reducing site) must be heavily buffered, with limited proton access.

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NITROGENASE REACTIVITY USING TI(III) AS REDUCTANT

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During Nitrogenase catalysis using dithionite, the Fe protein exclusively utilizes the $[Fe_4S_4]^{2+}$/ $[Fe_4S_4]^{1+}$ redox couple to transfer a single electron to the MoFe protein. The report that the Fe protein can exist as $[Fe_4S_4]^0$ and that this redox state is functional raises the possibility that an alternate Fe protein cycle utilizes the $[Fe_4S_4]^{2+}$/ $[Fe_4S_4]^0$ redox couple (Watt, Reddy, 1994). Seefeldt and Ensign (1994) found that Ti(III) an effective reductant for nitrogenase catalysis. However, they only obtained data using the $[Fe_4S_4]^{2+}$/$[Fe_4S_4]^{1+}$ redox couple because optical assays were performed at relatively low Ti(III) concentrations. We have used a variety of Ti(III) sources and report the results including specific activity curves for both the Fe protein and MoFe protein. Ti(III) was found to be quite inhibitory at lower concentrations than dithionite and we have identified the inhibiting species.

Because H_2 reduced Ti(III) is no longer available a variety of Ti(III) sources were investigated. We determined that the anaerobically evaporated aqueous $TiCl_3$ was the most active Ti(III) source. Using aqueous Ti(III) citrate, MoFe specific activities were comparable to dithionite at a 5:1 Fe:MoFe ratio. ATP/2e values of 2 were obtained at high [Ti(III)] while values of 5 were obtained at low [Ti(III)] (< 3 mM). This occurs because the Ti(III) citrate cannot maintain the $[Fe_4S_4]^0$ redox state at low [Ti(III)]. The reduction of 15 μM $[Fe_4S_4]^{1+}$ using 0.5-5 mM Ti(III) is first order in both $[Fe_4S_4]^{1+}$ and [Ti(III)] with an overall second order rate constant of ~66 M^{-1} s^{-1} at 30 °C. The reduction of $[Fe_4S_4]^{2+}$ has the same rate constant because reduction of $[Fe_4S_4]^{1+}$ is rate limiting. Ti(III) is inhibitory at much lower concentrations than dithionite. Assays performed using 7.6 mM Ti(III) demonstrated that activity deviated from linearity once 10% of the Ti(III) had been used. The citrate, used to keep the Ti(III) in solution, is about 2-3 times more concentrated than Ti(III). When Ti(IV) and citrate is added to dithionite assays inhibition is observed. Both citrate and Ti(IV) inhibit nitrogenase activity. At 7 mM Ti(III) the MoFe activity curve has a slightly slower rate than the 2 mM curve, but the overall maximum specific activity is the same (~1,800 nmol min^{-1} mg^{-1}). The Fe protein activity curve for 1 mM Ti(III) and 7 mM Ti(III) are different. As with dithionite at MoFe:Fe ratios above 2 with 1 mM Ti(III) MoFe inhibition was observed. At 7 mM Ti(III), Fe specific activities approach 3,400 nmol min^{-1} mg^{-1} at MoFe:Fe ratios of 12. At 7 mM Ti(III) MoFe inhibition is not observed. We attribute the increase in Fe protein activity to the operation of the $[Fe_4S_4]^{2+}$/ $[Fe_4S_4]^0$ redox couple.

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RE-EVALUATION OF THE KINETICS OF NITROGEN FIXATION

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Although nitrogen fixation using the Haber method is an expensive process requiring high temperature and pressure, certain organisms are able to fix nitrogen at atmospheric pressure and room temperature. These organisms contain two dissimilar proteins, the iron protein (Fe protein) and the molybdenum-iron protein (MoFe protein), that catalyze this reaction. The mechanism by which these proteins interact is at the core of understanding the *in vivo* process of nitrogen fixation. The current Thomeley and Lowe model of nitrogen fixation requires that the Fe protein repeatedly transfers one electron at a time to the MoFe protein, each interaction being identical, until sufficient electrons have been collected by the MoFe protein to reduce the desired substrate (Thomeley, Lowe, 1985).

The alternative mechanism for the Fe protein-MoFe protein allows for different interactions between the two proteins and the possibility of a doubly reduced iron protein being

$$[\mathrm{Fe}] + [\mathrm{MoFe}] \xleftrightarrow{K_1} [\mathrm{MoFe(Fe)}]$$

$$[\mathrm{MoFe(Fe)}] + [\mathrm{Fe}] \xleftrightarrow{K_2} [\mathrm{MoFe(Fe)_2}]$$

$$[\mathrm{MoFe(Fe)_2}] \xrightarrow{k_3} [\mathrm{MoFe}] + 2[\mathrm{Fe}] + \mathrm{Products}$$

$$\mathrm{Rate} = \frac{K_1 k_3 [\mathrm{MoFe}][\mathrm{Fe}]^2}{K_1[\mathrm{Fe}]^2 + K_1 K_m [\mathrm{Fe}] + K_m}$$

the reductant *in vivo* according to the equation and mechanism given above. This new model attempts to fit the nitrogenase “dilution effect”, “MoFe inhibition”, and sigmoidal activity curves using a single model whereas three separate explanations were required previously.

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CHARACTERIZATION OF THE E146D FE PROTEIN MUTANT OF *AZOTOBACTER VINELANDII:* FUNCTION IN NITROGENASE TURNOVER, FEMO COFACTOR BIOSYNTHESIS AND INSERTION

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The Fe protein of nitrogenase has at least three functions: electron transfer to the MoFe protein, the initial biosynthesis of the FeMo cofactor and the insertion of the preformed FeMo cofactor into a FeMo cofactor-deficient MoFe protein synthesized by ***ΔnifH*** strains. The exact functions of the Fe protein in the FeMo cofactor synthesis and insertion reactions are currently not known. Numerous Fe protein mutants, which are unable to support nitrogen fixation, have been described and sequenced over the years. Using this information we searched for mutations that did not involve residues that appeared to be involved in nitrogenase turnover. We hoped to find residues in portions of the Fe protein that were critical for FeMo cofactor biosynthesis or insertion. We identified the conserved E146 as a possible candidate. Therefore we constructed an E146D variant of the Fe protein and examined its function in nitrogenase turnover, FeMo cofactor biosynthesis and insertion.

The E146D surface residue mutation of the Fe protein affects the ability of *A. vinelandii* to grow under nitrogen fixing conditions. The doubling time is 50% compared to wild-type. However, purified Fe protein shows almost wild-type H_2 and C_2H_4 evolution activity. Typical MgATP and MgADP induced conformational changes occur as observed by EPR and behavior towards iron chelators. Like wild type Fe protein, E146D Fe protein can exist in an oxidized +2, reduced +1 and superreduced 0 state. The MoFe protein of *A. vinelandii* E146D NifH is partially FeMo cofactor deficient. Therefore, it shows only around 55% H_2 and C_2H_4 evolution activity compared to wild-type. This is consistent with its observed intensity of the FeMo cofactor typical S=3/2 EPR signal, the molybdenum and iron content.

The E146D Fe protein is defective in the insertion of isolated FeMo cofactor into FeMo cofactor-deficient MoFe protein of ***ΔnifH*** strain DJ54. It is particularly inefficient in the initial period of the insertion reaction. After this barrier is overcome, the insertion reaction can carry on at a higher rate.

The data in the present study indicates that the Fe protein contains distinct domains that are required for its various functions. The conserved E146 seems to be located in a domain necessary for the FeMo cofactor insertion reaction. That reaction is known to require the Fe protein, MgATP, the FeMo cofactor-deficient MoFe protein and at least one other protein. Because it is a surface mutation, E146D Fe protein may have difficulties interacting with either the FeMo cofactor-deficient MoFe protein or the other protein(s) required for this reaction.

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SUBSTRATE-REDUCTION PROPERTIES OF THE $\alpha195^{ASN}$ *A. VINELANDII* NITROGENASE MoFe PROTEIN

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Substitution of the $\alpha195^{His}$ residue results in a variety of altered MoFe proteins, none of which is able to reduce N_2 at appreciable rates. The crystal structure shows that $\alpha195^{His}$ is not covalently linked to FeMo-cofactor but is close enough to form a hydrogen bond to one of its sulfides. Analysis of the catalytic and spectroscopic properties of $\alpha195^{His}$ altered MoFe proteins has led to the proposal that $\alpha195^{His}$ helps to correctly position the FeMo-cofactor within the MoFe protein such that N_2 binding can occur (Kim et al., 1995; DeRose et al., 1995). The $\alpha195^{Asn}$ MoFe protein is of particular interest because it retains the ability to catalyze the reduction of H^+ to H_2 and C_2H_2 to C_2H_4 with accompanying C_2H_6.

We present data to show that 101kPa N_2 results in a 30% inhibition of H_2 evolution and an uncoupling of electron transfer from ATP hydrolysis (ATP:$2e^-$ = 7.2:1) and also inhibits C_2H_2 reduction. N_2 binds reversibly to the $\alpha195^{Asn}$ MoFe protein but is not reduced to NH_3. Although H_2 reverses the N_2 inhibition of C_2H_2 reduction, N_2-catalyzed HD formation does not occur. Based on K_m determinations, C_2H_2 was a poorer substrate for the $\alpha195^{Asn}$ MoFe protein than for the wild-type MoFe protein. The $\alpha195^{Asn}$ MoFe protein produces 23% of total product from C_2H_2 as ethane, 46% as ethylene and 31% as H_2. When C_2D_2 is the substrate, 37% of the $C_2H_2D_2$ is the *trans*-isomer (*c.f.* 4% for wild-type), suggesting a correlation between the ability to produce C_2H_6 from C_2H_2 and the loss of stereospecificity of acetylene reduction to C_2H_4. *Trans*-$C_2H_2D_2$ is not epimerized by turning over wild type or $\alpha195^{Asn}$ MoFe protein, implying that the intermediate in C_2H_2 reduction, which leads to *trans*-$C_2H_2D_2$, is not accessible to exogenous C_2H_4. Ethylene inhibits total electron flux, without uncoupling electron transfer from ATP hydrolysis, for both the $\alpha195^{Asn}$ and wild-type MoFe proteins. Ethylene is a somewhat better substrate for the $\alpha195^{Asn}$ MoFe protein, which exhibited a K_m of 48kPa and a specific activity for C_2H_6 formation approximately 10-fold higher than wild type. These results suggest that the C_2H_4-reduction site is affected by substitution at the $\alpha195$ residue, although whether the effect is related to the substrate-reduction site directly or is mediated through disturbance of the delivery of electrons/protons is unclear. A scheme encompassing these data is presented.

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STUDIES ON A PROTEOLYTIC ACTIVITY TOWARDS NITROGENASE IN *Rhodospirillum rubrum*

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1. Introduction

In the photosynthetic bacterium *Rhodospirillum rubrum* nitrogen fixation is regulated at the metabolic level in addition to the transcriptional control operating in all diazotrophs studied. However, little is known about the turn-over of nitrogenase and how this process is regulated. A recent report shows that in the unicellular cyanobacterium *Gloeothece* a proteolytic activity is appearing/disappearing in correlation to the dark/light cycle and results in complete degradation of nitrogenase early in the light phase (Reade et al 1999). In this study we have investigated degradation of nitrogenase in *R.rubrum.*

2. Experimental

R.rubrum was grown photoheterotrophically, either with NH_4^+ or N_2 as nitrogen source, and with 20 mM D,L-malate as carbon source. Extracts were produced by osmotic shock and chromatophores removed by centrifugation. Degradation of nitrogenase was studied by incubating extracts under different conditions followed by SDS-PAGE/Western blotting analysis.

3. Results & Discussion

In *R. rubrum* a decrease in the amount of nitrogenase is apparent as the culture enters stationary phase, which is due to both repression of transcription and proteolysis. We could show that in extracts nitrogenase was degraded by a proteolytic activity that could be specific for nitrogenase, as e.g. glutamine synthetase was not degraded nor any other protein as detected by silver staining of SDS-PAGE gels. The activity seems to be dependent on the presence of oxygen, no significant proteolysis of nitrogenase was demonstrated under anaerobic conditions. Inhibitor studies indicate that the activity was due to a serine protease, but do not exclude the involvement of more than one protease. As degradation of nitrogenase *in vivo* is increased when the pool of reductant for nitrogenase is exhausted, it is possible that the redox state of nitrogenase is important for its function as a substrate to the protease(s) involved.

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A NEW ROUTE TO THE SYNTHESIS OF TRINUCLEAR COMPLEXES CONTAINING V^{II} AND FE^{II}

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The trinuclear complex $[V_3(\mu\text{-Cl})_3(\mu_3\text{-Cl})_2(tmeda)_3](BPh_4)$ (A) was the only product isolated from the reaction of $[V_2Cl_3(thf)_6](BPh_4)$ with excess of *N,N,N',N'*-tetramethylethylenediamine (tmeda) in thf, through a mechanism which probably involves partial breakage of the binuclear complex into mononuclear species such as "$VCl_2(tmeda)_n$" which react in a further step with the intact molecules of the binuclear complex. The only reaction known so far to prepare (A) involved the reaction of $[VCl_2(tmeda)_2]$ with $NaBPh_4$ through a *halide abstraction pathway.* To establish the *new route* mechanism, we conducted the reaction between the well characterized mononuclear blue complex $[VCl_2(tmeda)_2]$ and the green binuclear compound $[V_2(\mu\text{-Cl})_3(thf)_6](BPh_4)$ in the presence of tmeda (1:1:1) in refluxing thf. After slow diffusion of a hexane layer, the resultant turquoise solution afforded large deep green prisms formulated as (A) in accordance with the analytical data. Found (%): C, 50.78; H, 6.86; N, 8.42; Calculated: C, 50.54; H, 6.88; N, 8.42. The molecular ion for $[V_3Cl_5(tmeda)]^+$ (m/z 678) was also present in the FAB mass spectra of (A). The ^{1}H-NMR spectrum of the product in thf-d_8 solution shows broad signals in the range of 10.0-12.5 ppm due the paramagnetic effect of the V^{II} centres and attributed to the coordinated tmeda.

Our results indicate that this new approach might also be effective to the synthesis of heterobimetallic trinuclear compounds with the formulae, $[M^{II}_{(3-x)}M'^{II}_{x}(\square\text{-Cl})_3(\square_3\text{-Cl})_2L_{(3-x)}L'_x]$, (M and M = transition metals, and L and L = bidentate ammines). This work is currently under way in our laboratory. We have prepared $[V_2Fe(\square\text{-Cl})_3(\square_3\text{-Cl})_2(tmeda)_3](BPh_4)]$ through the reaction between $[V_2(\square\text{-Cl})_3(thf)_6](BPh_4)$ and $[FeCl_2(tmeda)_2]$ in thf. The product has not been fully characterized yet, but the elemental analysis is very promising: Found (%): C, 48.90; H, 7.04; N, 7.03; Fe, 9.30; Calculated: C, 50.30; H, 6.84; N, 8.38; Fe, 9.36. The product exhibits vibration modes with energies, which are close but undoubtedly different from those seen in the relative homometallic trinuclear complexes. The new route allows a better control of the reaction pathway, reducing the formation of undesired side products. It is certainly a simple and convenient alternative to the known Halide Abstraction Route.

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COMPLEXES OF $V^{III/IV}$ WITH DEPROTONATED ALDITOLS AND MONOSACCHARIDES

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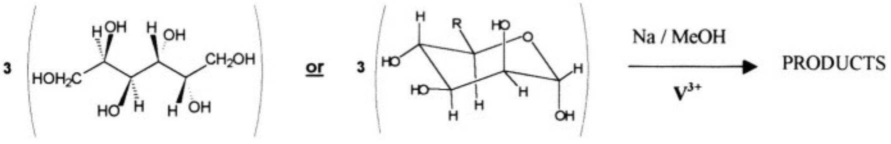
Interest in the chemistry of $V^{II/III}$ species supported by O-donor ligands has grown since the discovery of catalytic or stoichiometric reduction of N_2 by V^{II}-catecholates and -hydroxides (Bazhenova, Shilov 1995). Alternatively, medium/high oxidation state V-alkoxides have been seen as potential molecular precursors of poly(hetero)metallic oxides with applications in sol-gel processes (Mehrota, Singh 1998). Our work investigates the reactions of V^{III}-starting materials with sterically-demanding alkoxides generated from alditols and saccharides. Our general aims are the preparation of coordination compounds of vanadium(III/IV) with O-donor ligands, reduction of the reaction products, and analysis of experimental conditions that can promote an interaction with N_2.

Syntheses were carried out in refluxing methanol under N_2 using Schlenk techniques. V^{III}-starting materials ($[V(acac)_3]$ and $[VCl_3(thf)_3]$; acac = acetylacetonate; thf = tetrahydrofuran) and the *in-situ* generated di-sodium salts of galactitol (dulcitol, $dulc^{2-}$) or D-mannose (man^{2-}) were combined in a 1:3 ratio (scheme). Air-sensitive products were isolated and characterised by C, H and V analyses, ^{1}H-NMR, FTIR and EPR spectroscopies, as described below.



According to elemental analyses, the light-green product A obtained from $dulc^{2-}$ is mononuclear and contains three chelating $dulc^{2-}$ moieties bound to vanadium. EPR results suggest oxidation of V^{III} to V^{IV} during the reaction ($g_{iso} = 1.978$; $A_{iso} = 73.8 \pm 0.2 \times 10^{-4}\ cm^{-1}$; $g_{\parallel} = 1.959$; $g_{\perp} = 1.976$; $A_{\parallel} = 147.5 \pm 0.2 \times 10^{-4}\ cm^{-1}$; $A_{\perp} = 44.5 \pm 0.2 \times 10^{-4}\ cm^{-1}$). FTIR spectra indicate that A does not contain V=O . ^{1}H-NMR data support metal coordination through O-3 and O-4 ($\delta = 3.65\text{-}3.68$ ppm, 400 MHz in D_2O) and indicate the presence of non-coordinated methanol in the solid ($\delta = 3.29$ ppm).
The olive-green product obtained from $[VCl_3(thf)_3]$ and man^{2-} (B) apparently contains vanadyl ($\nu(V{=}O)$ 926 cm^{-1}). This is also supported by EPR data ($g_{iso} = 1.975$; $A_{iso} = 78.7 \pm 0.2 \times 10^{-4}\ cm^{-1}$; $g_{\parallel} = 1.958$; $g_{\perp} = 1.980$; $A_{\parallel} = 152.0 \pm 0.2 \times 10^{-4}\ cm^{-1}$; $A_{\perp} = 45.5 \pm 0.2 \times 10^{-4}\ cm^{-1}$). ^{1}H-NMR results suggest that man^{2-} is coordinated to V^{IV} through the oxygen atom bound to C-2 ($\delta = 4.112/4.120$ ppm, α-form; $\delta = 4.134/4.143$ ppm, β-form; 400 MHz in D_2O). O-3 is also probably involved in metal binding (Sreedhara et al 1994). Both anomeric forms of the monosaccharide bind the V ion. Magnetochemical analyses of A and B are under way to confirm the oxidation state of the metal ion in the complexes. Reduction of the products and studies of their reactivity towards N_2 follow.

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SECTION II

REGULATION OF NITROGEN FIXATION AND ASSIMILATION

REGULATION OF *NIF* GENE EXPRESSION IN FREE-LIVING DIAZOTROPHS: RECENT ADVANCES

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1. Introduction

It is now almost exactly twenty years since the first studies were carried out on the regulation of *nif* gene expression in *Klebsiella pneumoniae* (Dixon et al, 1980). Since that time investigations have been extended to a variety of free-living diazotrophs so that in a number of organisms we now have a good understanding of the major components required for activation of *nif* gene transcription and a reasonable understanding of the signal transduction pathways that ultimately regulate gene expression. The present challenges are firstly to understand the differences between organisms and how these allow adaptation to the specific physiological requirements of particular species, and secondly to describe the best characterised systems at the molecular level.

2. The *nif*-specific Activator NifA

Recent studies relating to the *nif*-specific activator NifA have been focussed on two areas; the structure of the protein and regulation of its activity. NifA belongs to a family of transcriptional activators all of which act in concert with an RNA polymerase holoenzyme containing the alternative sigma factor σ^N. The formation of open complexes and transcription initiation is catalysed by NifA and involves nucleoside triphosphate hydolysis. NifA and related activators have a well defined three domain architecture in which the amino terminal domain is a regulatory domain, the central domain is highly conserved and contains characteristic ATP binding motifs, and the carboxy terminal domain constitutes the DNA-binding domain. An understanding of the mechanism of action of NifA would undoubtedly be aided by structural information but the protein itself has in most cases proved extremely refractory to purification. Only the *Azotobacter vinelandii* protein has been purified in its native form (Austin et al, 1994). Three other NifA proteins have been purified in different forms: the *K. pneumoniae* protein has been purified as separate domains fused to the maltose-binding protein (Berger et al, 1995); the linked central and C-terminal domains of *Herbaspirillum seropedicae* NifA can be purified with an N-terminal His-tag and the protein binds specifically to the *H. seropedicae nifB* promoter *in vitro* (Monteiro et al, 1999); and *A. brasilense* NifA has been purified as a glutathione-S-transferase fusion protein but although proteolytic release gave partially soluble NifA it was inactive (Passaglia et al, 1998).

The central domain of NifA is sufficient by itself to activate transcription (Berger et al, 1994) and whilst structural data is still lacking, a model for the architecture of the central domain of NifA and related enhancer-binding proteins (EBPs) has been proposed that accommodates seven highly conserved regions (C1-C7) within this protein family (Osuna et al, 1997). Two members of the EBP family, *E.coli* TyrR and *Rhodobacter capsulatus* NtrC, are not **σ^N-dependent** activators and both have deletions within the C3 region as defined by Osuna *et al* (Osuna et al, 1997). Gonzalez *et al* (Gonzalez et al, 1998) have now demonstrated, using *B. japonicum* NifA, that a conserved hydrophobic patch (GAFTGA) in region C3 is involved in positive control and may be a

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recognition element for the RNA polymerase promoter complex. This proposal is consistent with the phenotypes of mutants of other EBPs, namley NtrC (North et al, 1996) and DctD (Wang et al, 1997), that also map to the C3 region.

The structure of the isolated C-terminal domain of *K. pneumoniae* NifA has been analysed by NMR, FT-IR and CD and shown to have a distinct folded structure which contains appreciable amounts of **α-helix** (Missaillidis et al, 1999). The data reported are consistent with the presence of a helix-turn-helix (HTH) motif as proposed by Drummond *et al* (Drummond et al, 1986) and suggest that the motif is stabilised by a third helix N-terminal to the HTH.

3. Regulation of NifA Activity in Response to Oxygen

The activity of NifA is usually regulated in response to two major environmental factors, oxygen and fixed nitrogen. NifA proteins in free-living diazotrophs can be divided into two classes, those whose activity is regulated by a sensor protein NifL encoded in the same operon as NifA, and those that are encoded in a monocistronic operon and where no NifL-like protein has been identified.

In those organisms that do not apparently have a NifL protein the activity of the NifA protein is inherently oxygen sensitive, a property that these proteins share with the NifA proteins of the symbiotic rhizobia (Fischer, 1994). In all these cases e.g. *A. brasilense, H. seropedicae, R. capsulatus* a cysteine motif $\mathbf{(Cys\text{-}X_{11}\text{-}Cys\text{-}X_{19}\text{-}Cys\text{-}X_4\text{-}Cys)}$ is present between the central domain and the DNA-binding domain. It has been suggested that this motif constitutes a binding domain for a metallocluster that mediates the redox sensitivity of these proteins (Fischer et al, 1988) and consistent with this is the observation that activity of the N-terminally truncated form of *H. seropedicae* NifA is Fe dependent (Souza et al, 1999). Until now the intractability of this class of NifA proteins has precluded any *in vitro* biochemical characterisation of this redox response but this may now be accessible in *H. seropedicae.* The preliminary data indicate that although the protein does not form open complexes, DNA-binding is not inhibited when both the binding experiments and the protein purification are performed in air (Monteiro et al, 1999). Similar results have been reported in band shift assays with *E.coli* extracts expressing *A. brasilense* NifA which showed binding to the *nifH* promoter in the presence of oxygen (Passaglia et al, 1998).

4. The NifL Protein

The NifL protein had until recently only been reported in *K. pneumoniae, A. vinelandii* and *Enterobacter agglomerans* all of which are in the γ proteobacteria but a *nifLA* operon has also now been identified in *Azoarcus* sp. BH72, a member of the **β** proteobacteria (Egner et al, 1998). Inhibition of NIFA activity by NIFL requires stoichiometric amounts of the two proteins, implying that the mechanism of inhibition is by direct protein-protein interaction rather than by catalytic modification of the NIFA protein. This interaction facilitates regulation of NifA activity in response to oxygen (or redox), fixed nitrogen and energy status, each of which is signalled by an independent pathway.

Both *AvNifL* and *Kp*NifL are flavoproteins with FAD as the prosthetic group (Hill et al, 1996;Schmitz, 1997). The FAD is bound to the N-terminal domain and the isolated domain has identical spectral features to the native protein (Söderbäck et al, 1998;Schmitz, 1997). Signal transduction to NifL may involve an Fe-containing protein (Henderson et al, 1989;Schmitz et al, 1996) although there may not be a specific electron donor *in vivo*. Studies of NifL reduction *in vitro* have used both the *E.coli* flavohaemoprotein Hmp and spinach ferredoxin:NAD(P) oxidoreductase (Macheroux et al, 1998). In these experiments re-oxidation of NifL occurs rapidly in the presence of air, raising the possibility that NifL might sense intracellular oxygen.

The NifL protein is probably folded into two domains tethered by a Q-linker and it has been proposed that the N-terminal domain is regulatory whilst the C-terminal domain shows some homology to the histidine protein kinase family of two-component regulatory proteins (Drummond et al, 1987). Recent recognition of more extensive homologies together with the solutions of crystal structures for a number of apparently related proteins has provided considerable data that pertains to NifL structure and function.

Zhulin *et al* (Zhulin et al, 1997) identified a conserved domain, termed the PAS domain, that is present in a large family of proteins many of which are involved in responses to oxygen, redox or light. These motifs have since been extended to a number of voltage-gated potassium channels and consequently designated LOV (light, oxygen, voltage) domains (Huala et al, 1997). The motifs are located in the N-terminal domain of the NifL proteins and crystal structures are now available for three proteins that contain such motifs: photoactive yellow protein (Pyp) (Pellequer et al, 1998), *B. japonicum* FixL (Gong et al, 1998) and the HERG potassium channel (Morais Cabral et al, 1998). These structures reveal a common fold in all three proteins, the dominant feature of which is a five-stranded antiparallel β barrel that has been described as forming a "left-handed glove". In FixL the glove encloses a haem cofactor whilst in Pyp it encloses a chromophore. The theme linking this domain family appears to be that they are all involved in signal transduction with the common fold being capable of accommodating a range of different cofactors. At least three proteins that contain PAS domains are flavoproteins, namely NifL and the *E.coli* aerotaxis protein Aer (Rebbapragada et al, 1997) in which the cofactor is FAD, and the *Arabidopsis* phototropism protein NPH1 which contains FMN (Christie et al, 1999). In both NifL and NPH1 the precise binding sites of the cofactors are still unknown but it is tempting to speculate that they also lie within the same fold. The recognition of this common structural theme in a family of proteins including NifL allows the development of likely structural models for at least part of the protein.

The inhibitory activity of NifL on NifA is stimulated by the presence of adenosine nucleotides and the C-terminal domain of *Av* NifL is protected from trypsin digestion in the presence of ADP, suggesting that ADP binding induces a conformational change in that domain (Soderback et al, 1998). Indeed adenosine nucleotides promote complex formation allowing isolation of the complex *in vitro* and experiments with truncated proteins demonstrate that the C-terminal domain of NifL and the N-terminal region of NifA potentiate the ADP-dependent stimulation of NIFL-NIFA complex formation (Money et al, 1999). Hence NifL may also sense the cellular energy status through the intracellular ATP/ADP ratio. Structural models for homologues of the C-terminus of NifL are also beginning to emerge with the solution of a crystal structure for histidine protein kinase CheA (Bilwes et al, 1999) and a NMR structure for the histidine kinase (HK) domain of EnvZ (Tanaka et al, 1998). Although the C-terminal domain of *A. vinelandii* NifL shows greater homology to the HK domain than it does to its *K. pneumoniae* counterpart, it is possible that both domains have similar structures and that the CheA and EnvZ domains provide good models; e.g. it is likely that NifL shares a similar nucleotide binding cavity to that described in CheA.

5. The Role of $\mathbf{P_{II}}$ Proteins in Nitrogen Control of *nif* Gene Expression

In many diazotrophs it is clear that not only is *nifA* expression controlled by the global nitrogen regulation (*ntr*) system but also the activity of NifA is responsive to the availability of fixed nitrogen. An obvious candidate for the signal transduction pathway to NifA was the global nitrogen regulation pathway encoded by the *glnD, glnB, ntrB* and *ntrC* gene products, but initial studies in *K. pneumoniae* indicated that neither GlnD or GlnB (P_{II}) played a direct role in NifL-mediated regulation of NifA activity in response to fixed nitrogen (Holtel et al, 1989;Edwards et al, 1995). However the discovery of a second gene (*glnK*) encoding a P_{II}-like protein in *E.coli* (van Heeswijk et al, 1996) offered another potential candidate.

We now know that two *glnB*-like genes are found in most of the proteobacteria, an exception being *A. vinelandii* which has only a *glnK* gene (Meletzus et al, 1998). The primary amino acid sequences of the GlnB and GlnK proteins from the same organism are typically about 70% identical and as expected the crystal structures of the two proteins are very similar (Carr et al, 1996;Xu et al, 1998). The similarities between the two proteins make the clear identification of a particular gene as being either *glnB* or *glnK* quite difficult. However five potentially diagnostic residues (residues 3,5,52,54 and 64) have been described (Jack et al, 1999) and comparative genomic analysis indicates that GlnK proteins are almost invariably encoded in an operon together with the ammonium transporter gene *amtB* (Thomas et al, 1999). The physiological role of GlnK has yet to be clearly defined though the linkage of *glnK* and *amtB*, even in organisms that lack *glnB* and the *ntr* system, suggests that its primary function may either be in regulation of AmtB activity or in a signal transduction pathway that involves AmtB (Thomas et al, 1999). Studies in *E.coli* indicate that in some but not all situations GlnK can substitute for GlnB (Atkinson et al, 1998). The identification of distinct roles for each protein is considerably complicated by recent demonstrations that, at least in *E.coli,* the two proteins can also from heterotrimers (Forchhammer et al, 1999)(van Heeswijk, unpublished).

The first indications that P_{II}-like proteins might indeed be involved in the regulation of NifA activity in response to cellular nitrogen status came from the observation that in *A. brasilense* a *glnB* mutant was Nif$^-$ (Liang et al, 1992). As *nifA* expression was not controlled by GlnB (P_{II}) it appeared that P_{II} is required to maintain the active form of *A. brasilense* NifA and a similar situation is found in *H. seropedicae* where a *glnB* mutant is again Nif$^-$ (Benelli et al, 1997).

Studies of the *H. seropedicae* NifA protein *in vivo* show that the full-length protein expressed in *Azospirillum brasilense* is active only under low oxygen tensions and in the absence of ammonium, but the protein is not active when expressed in *E.coli* or *K. pneumoniae* (Souza et al, 1999). By contrast, the N-terminally truncated form is still active in the presence of ammonium and is also active in *E.coli* or *K. pneumoniae* (Souza et al, 1999;Monteiro et al, 1999) although the N-terminal domain expressed *in trans* can inhibit activity of the truncated protein (Monteiro et al, this volume). A role for the regulatory domain in nitrogen sensing has also been identified in *A. brasilense* (Arsene et al, 1996), *Rhodobacter capsulatus* (Paschen et al, 1998) and the alternative activators VnfA and AnfA (Drummond et al, 1995). The inactivity of *H. seropedicae* NifA in *E. coli* may reflect interspecific P_{II} differences such that *E.coli* P_{II} is unable to “activate” the *H. seropedicae* NifA (Souza et al, 1999). In summary whilst many details remain to be elucidated, some elements of a common theme involving interaction between a P_{II}-like protein and the amino terminal domain of NifA are emerging in those organisms that do not have a NifL protein. An exception to this theme is *Azorhizobium caulinodans* where single *glnB* or *glnK* mutants behave as wild-type and show ammonium repression of nif expression but the *glnBglnK* double mutant is derepressed in ammonium (Michel-Reydellet et al, 1999).

In *K. pneumoniae* and *A.vinelandii* the modulation of NifA activity in response to the cellular nitrogen status involves NifL suggesting that NifL could act as a nitrogen sensor or that complex formation between NifL and NifA might facilitate nitrogen control by regulating access of other signal proteins to NifA. Genetic studies indicate that the *K. pneumoniae* NifL/NifA interaction is modulated by GlnK in such a way that in N-limiting conditions GlnK is required to relieve the inhibitory effect of NifL on NifA (He et al, 1998;Jack et al, 1999). As predicted by Edwards and Merrick (1995) the effect of GlnK is independent of its uridylylation state which raises the question as to how NifL inhibition is rapidly re-established in response to elevated N-status. Studies of the *K. pneumoniae* NifL/NifA interaction in *E.coli* indicate that GlnB can counteract the positive action of GlnK and it is possible that this negative effect might be mediated by GlnK/GlnB heterotrimer formation (Arcondeguy *et al.* this volume). In *A. vinelandii glnD* (*nfrX*) mutants are Nif^- and this phenotype can be suppressed by mutations in *nifL* suggesting that UTase may be required to maintain NifL in an inactive from under conditions of nitrogen limitation (Contreras et al, 1991). However the activity of NifL does not apparently correlate with the uridylylation state of $\mathrm{P_{II}}$ (Rudnick et al, 1998). The *A. vinelandii* NifL protein responds to the availability of fixed nitrogen when expressed in *E*.coli (Soderback et al, 1998) and recent studies *in vivo* and *in vitro* indicate that whilst neither GlnB nor GlnK are required for derepression, the inhibitory function of *A. vinelandii* NifL is activated under N-excess conditions through interaction with either of the non-modified forms of *E.coli* GlnB or *A. vinelandii* GlnK (Reyes-Ramirez *et al.* this volume).

To date the studies with *K. pneumoniae* and *A.vinelandii* do not indicate whether GlnK interacts primarily with NifL or with NifA. The present data would be consistent with a model proposed by Drummond *et al* (Drummond et al, 1990) which requires both NifL and NifA to be in a “derepressed” conformation to relieve mutual binding. In this case the primary interaction of GlnK could be with NifA which would have the merit of offering some uniformity to the mechanism of controlling NifA activity in all free-living diazotrophs.

6. Conclusions

The prospects for a detailed understanding of many aspects of *nif* regulation at the molecular level are now extremely good with progressively more aspects of the system becoming tractable *in vitro*. Structural information on homologous systems is offering new insights into mechanistic aspects of these processes and, as the history of this field of research clearly shows, many of these studies continue to have major implications in other areas of prokaryotic biology.

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ACTIVATION OF TRANSCRIPTION BY THE SIGMA-54 RNA POLYMERASE HOLOENZYME

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1. Introduction

The bacterial sigma-54 protein associates with core RNA polymerase to form a holoenzyme that functions in enhancer dependent transcription. Isomerisation of the sigma-54 polymerase and engagement with melted DNA in open promoter complexes requires nucleotide hydrolysis by enhancer binding activators such as NifA, NtrC and DctD. Here, we describe properties of mutant forms of sigma-54 that allow transcription *in vitro* without activator. Such mutants may be defective in interactions that are normally changed by activator as part of the reaction pathway that leads to formation of the open promoter complex. We show that a single amino acid substitution in the *Klebsiella pneumoniae* sigma-54 C-terminal DNA-binding domain allows holoenzyme to isomerise, engage with stably melted DNA and to transcribe from transiently melting DNA without activator. The activator independent phenotype of the DNA-binding domain mutation appears largely indistinguishable from that arising through mutating amino terminal sigma Region I sequences. Thus two distinct sequence determinants in sigma-54 function for enhancer responsiveness. Results of experiments with partly melted promoter DNA templates sequences show that activator re models the sigma-DNA complex in a reaction that occurs with same requirements as does transcription activation, but which can proceed in the absence of the core polymerase subunits. We suggest that movements in Region I and the DNA-binding domain of sigma-54 facilitated by activator are required to allow the polymerase to isomerise.

In bacteria, two types of transcription initiation occur. One involves the RNA polymerase holoenzyme containing a sigma-70-type subunit, the other holoenzyme with the sigma-54-type subunit (Merrick, 1993). The sigma-70 holoenzyme allows DNA melting, to reveal the template strand, and the initiation of RNA synthesis without a requirement for remote enhancer elements. The sigma-54 holoenzyme employs specialised enhancer bound activator proteins such as NifA that catalyse holoenzyme post binding steps to accelerate the stable association of holenzyme with melted DNA near the transcription start site (Popham *et al*., 1989). The sigma-54 system can be regarded as sharing properties with eukaryotic enhancer dependent transcription.

The mechanism by which sigma-54 controls properties of the RNA polymerase core enzyme is of interest for determining at which steps activators accelerate transcription initiation. Current models for activation of the sigma-54 holoenzyme indicate that DNA melting *per se* is not rate limiting for initiation. Premelted promoter DNA does not overcome activator and nucleotide requirements for transcription (Wedel, Kustu, 1995; Cannon *et al.,* 1999). Analysis of mutations in sigma-54 Region I sequence suggests that the sigma amino terminal domain functions to inhibit isomerisation of the RNA polymerase, thus preventing the interaction with melted DNA necessary for initiation of RNA synthesis (Wang *et al.,* 1995; 1997a; Syed, Gralla, 1998; Cannon *et al*., 1999). Whether polymerase isomerisation precedes, is concomitant with or follows DNA strand separation is currently unknown.

Sigma-54 amino terminal Region I sequences residues 1-56 bind to core RNAP and may therefore control properties of the holoenzyme important for enhancer responsiveness (Gallegos *et al*.,

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1999a,b). The solvent accesibility of sequences within the C-terminal sigma-54 DNA-binding domain is changed in the holoenzyme when Region I is deleted, implying that Region I contributes to physical properties of the holoenzyme, some of which involve sequences that are closely associated with the DNA-binding function of sigma-54 (Casaz and Buck, 1997; 1999a). In initial complexes formed between holoenzyme and promoter DNA, sigma-54 contacts DNA that is melted out in open complexes, suggesting that the sigma DNA-binding domain influences open complex formation (*Cannon et al.,* 1995).

Here we describe (a) properties of mutant sigma factors that greatly alter the enhancer responsiveness of the holoenzyme and (b) a reaction between sigma, DNA and activator that leads to the conclusion that sigma-DNA complexes are remodelled to allow polymerase isomerisation and hence activated transcription.

2. Activator-Independent Transcription Activity of sigma-54 R336A and Region I Mutants

Sigma-54 dependent transcription from the *Sinorhizobium meliloti nifH* promoter was measured *in vitro* using supercoiled DNA. Using a preincubation of template DNA holoenzyme and GTP prior to addition of other NTPs and heparin to destroy residual closed complexes we found that the DNA-binding domain mutant R336A allowed the holoenzyme to initiate transcription efficiently without activator (Figure 1). Under the same conditions, the wild type sigma-54 did not transcribe (Chaney and Buck, 1999).

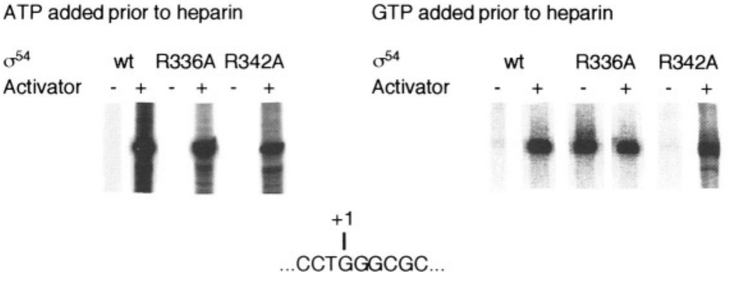

Figure 1. *In vitro* transcription at supercoiled *S. meliloti nifH* promoter. Holoenzyme formed with sigma-54 R336A transcribes without activator in the presence of initiating nucleotide GTP. The wild-type sigma or a mutant (R442A) showing like R336A reduced DNA binding relies upon activator and nucleotide hydrolysis for transcription (ATP lanes). Activator was PspFΔHTH (Casaz, Buck,1997).

Previously activator independent transcription has been described for mutants in the sigma amino terminal Region I sequence (Figure 2). To be absolutely sure that our R336A protein preparations were physically intact, they were purified as amino terminal His-6 fusions to preserve amino terminal sequences and confirmed by electrospray mass spectrometry. R336A holoenzyme was activated by **PspFΔHTH,** but needed 1.5-2 fold more activator for full transcription activity (Figure 1).

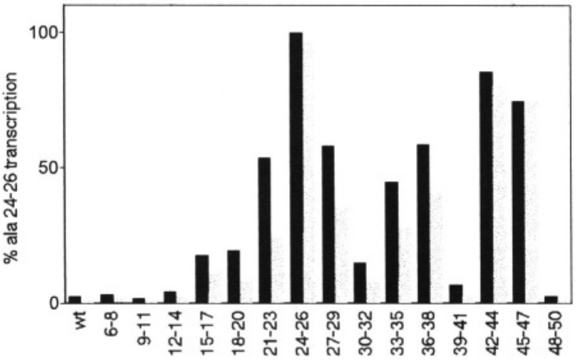

Figure 2. **Substitutions throughout Region I of sigma-54 allow transcription without activator (Casaz *et al.*, 1999b). Solid bars, with GTP; hatched, with GTPγS. The use of GTPγS establishes transcription is without activator nucleotide hydrolysis.**

3. Activator-Dependent Transcription Activity of Region I Mutants

Region I mutants are defective *in vivo* and *in vitro* for activated transcription (Figure 3) but are partially rescued *in vitro* by higher activator concentrations. The R336A mutant was similarly rescued (data not shown). It appears Region I and R336 set the apparent affinity of the holoenzyme for activator. Since no Region I mutant that allows activator independent transcription is fully intact for activated transcription, the determinants for each appear linked. Furthermore the extensive sensitivity of Region I to mutagenesis (36/50 residues) suggests that several different Region I dependent interactions are involved in activation. These likely include interactions with core, with the

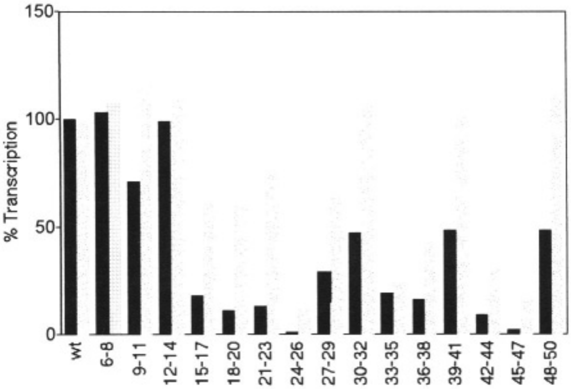

Figure 3. **Substitutions throughout Region I of sigma-54 allow diminish transcription with activator (Casaz *et al.*, 1999b). Solid bars, 4 μM activator; hatched bars, 100 nM activator.**

sigma DNA binding domain and with melted DNA at -12, although the last may be indirect (see below).

4. Sigma-premelted DNA Template Reactivities

Using an artificial bubble between -12, -11 to mimic the normal structural distortion seen in sigma-54 holoenzyme closed promoter complexes, we have shown that a new complex can form either in a reaction which requires activator and a hydrolysable nucleoside triphosphate or simply through re-binding sigma Region I provided *in trans* (Figure 4). We infer that activator functions to remodel the sigma DNA-complex in a reaction showing all the requirements needed for activated transcription: activator, NTP, Region I, and a nucleated state of the DNA associated with melting.

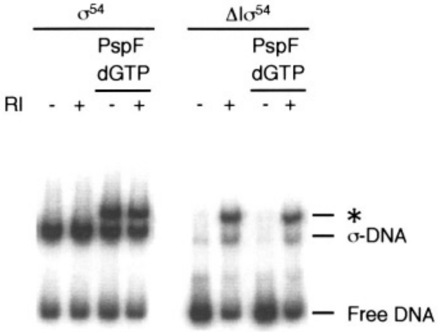

Figure 4. A: a new complex (*) between sigma-54 and bubble DNA is formed through the action of activator and nucleotide hydrolysis. B: a similar complex forms when rebinding of Region I to truncated sigma occurs in the presence of bubble DNA. ΔIσ^{54} lacks amino acids 1-56; RI comprises residues 1-56.

5. Overview

Results with the R336A mutant and Region I mutants provide evidence that sequences in the C-terminal and N-terminal parts of sigma-54 contribute to establishing the enhancer responsiveness of the sigma-54 holoenzyme. It appears that the substitutions allow the holoenzyme to adopt a conformation that allows it to stably engage with premelted DNA or to transcribe from transiently melting DNA. Results suggest that the holoenzyme has isomerised to reveal a single strand DNA-binding activity that allows interaction with melted DNA. In the two step model for activation at sigma-54 promoters (Wang, Gralla, 1996) , both Region I and R336 contribute to inhibiting polymerase isomerisation in the absence of activator and so prevent the holoenzyme making stable complexes with melted DNA (Chaney, Buck, 1999). The shared property could result from Region I controlling the conformation of sigma C-terminal sequences. Consistent with this proposal is the finding from protein footprints that the conformation of C-terminal parts of sigma are changed in the holoenzyme when Region I is deleted (Casaz, Buck, 1999a). Potentially a critical sigma-DNA interaction may be required to keep the polymerase silent, and this might be mediated both by Region

I and the DNA-binding domain of sigma-54. The ability of the sigma-bubble complex to undergo an activator dependent change (Figure 4) argues that for the normal reaction pathway to transcription to occur, activator alters Region I dependent interactions. Such activator dependent changes appear to depend critically upon promoter conformation, and suggest that sequences near -12 are especially important. The binding of Region I to core polymerase, its involvement in -12/-11 bubble template recognition and in sigma DNA-binding domain conformation indicate that a network of interactions controlled by Region I is changed to allow polymerase isomerisation and to reveal the single strand DNA-binding activity of the holoenzyme. The extent to which sigma-54 directly contributes to a ssDNA-binding activity remains to be determined. However it is clear that Region I stabilises holoenzyme on melted DNA (Gallegos *et al.*, 1999b; Gallegos *et al.,* this volume) raising the possibility some determinants for ssDNA binding are in Region I. The contact sites between sigma-54 and activator required for catalysing polymerase isomerisation remain to be precisely defined (Lee, Hoover, 1995; Wang *et al.*, 1997b).

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NEW MECHANISMS OF BACTERIAL GENE REGULATION IN A NITROGEN-FIXING PHOTOTROPH

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1. Introduction

Rhodobacter capsulatus is an alpha proteobacterium that grows photosynthetically and can fix nitrogen. The two-component nitrogen regulatory (*ntrB/ntrC*) system in *R. capsulatus* is similar to the enteric counterpart from the standpoint of sensing and phosphorelay. However, the RcNtrB-phosphorylated RcNtrC activates the nitrogen-fixing regulatory cascade by a mechanism that has some properties of the bacterial enhancer binding proteins, which activate RNA polymerase/sigma N from a distance, and some of the classic bacterial activators, like CAP, which activate the housekeeping RNAP. Thus, the RcNtrC activation system could represent a transition between the two general types of bacterial activation systems. The *Rhodobacter* oxygen-sensing activators (RegA/B) and repressor (CrtJ) of photosynthesis genes also appear unique to alpha proteobacteria. The properties of these activation systems and some qualities of the Rc RNA polymerase are described within a general overview of related promoters and RNA polymerases.

2. Transcription Control in *R. capsulatus* Compared to That in *E.coli:* Mechanisms of the *Rhodobacter* RcNtrC and Photosynthetic Control Systems.

A database of promoters from the most studied gamma proteobacteria, *E. coli, Salmonella*, and *Klebsiella* has been compiled (Gralla, Collado-Vides, 1996). From approximately 150 different promoters and the location on the DNA where regulatory proteins bind, some general principles have emerged. Repressors typically bind within the location where the RNA polymerase (RNAP) holoenzyme binds, downstream of -35 (relative to the transcription start site). This "exclusive zone of repression" almost certainly reflects the mechanism of repression, involving competition with RNAP for binding to the promoter. In *R. capsulatus,* the genes for photosynthesis are only expressed anaerobically (Bauer, Bird, 1996). Some photosynthetic genes appear repressed by an activator (CrtJ) under aerobic conditions. Two such promoters are *bch* (for bacteriochlorophyll biosynthesis) *and puc* (encoding light-harvesting II apoproteins). We have shown that purified CrtJ represses σ^{70} RNAP transcription at the *bch* promoter by competiting with RNAP for the promoter (Bowman *et al.,* 1999). The *puc* promoter, unlike *bch,* is activated by the two component response regulator called RegA, which is phosphorylated by RegB in response to anaerobicity. Surprisingly, the *puc* promoter is repressed by CrtJ not by competition with RNAP binding but by competition with RegA for its binding site just upstream of –3 5 (see below).

Another lesson from the *E. coli* promoter database concerns two distinct mechanisms of activation. Promoters that are recognized by RNAP containing σ^{70} and those by the alternative σ^{54} RNAP showed distinct positions on the DNA where the activator proteins bind. All σ^{70} RNAP activators bound at -70 or closer, although for some promoters multiple binding sites were additionally located further upstream. This requirement for adjacent binding of activator and σ^{70} RNAP probably reflects activation by the activator protein interacting with the α and/or σ subunit of RNAP, thus, "recruiting" RNAP to the promoter (Ptashne, Gann, 1997) . Often, these promoters have poor -35 hexamers and the activator:RNAP interaction increases promoter occupancy by RNAP. In *R. capsulatus,* the photosynthetic activator RegA functions in this manner. Under anaerobic conditions, RegA is

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phosphorylated by the histidine kinase, RegB. RegB interacts with the cytochrome *c* oxidase such that electron flux to oxygen is sensed and this information is relayed to RegB (Oh, Kaplan, 1999). The phosphorylation of RegA by RegB, when no flux is sensed, results in activation of photosynthetic genes under anaerobic conditions. Important questions concern whether activation of these promoters by RegA is direct and if so, what RNAP holoenzyme(s) is used and what mechanism. *In vitro* transcription studies on the *puc* promoter proved that phosphorylated RegA directly activates *puc* transcription. Using different σ^{70}/RNAP concentrations, this was shown to occur by recruitment of RNAP to the promoter, which has a poor -35 hexamer. *In vivo* results had indicated that the *puf* operon, encoding reaction center and light harvesting apoproteins, requires RegA for activation but does not use the CrtJ repressor. *In vitro* reconstitution results have confirmed this. Although the *puf* promoter was suggested to use an alternative sigma factor, based on lack of consensus in the -35 and - 10 regions, *in vitro* reconstitution of *puf* with pure RegA and B and RNAP containing recombinant σ^{70} subunit established that the housekeeping RNAP is used (Bowman *et al.,* 1999).

In the *E.coli* database of promoters, regulatory proteins that bind to sites further upstream than -70, without other proximal sites, were limited to activators of the σ^{54} RNAP. These activators are also called enhancer-binding proteins (EBPs) and indeed function by a completely different mechanism than most proximally-binding activators. The bacterial regulator NtrC from gamma proteobacteria has been a model for this type of activator. No recruitment of σ^{54}/RNAP to the promoter appears to be necessary. Activation requires ATPase activity by the regulator. The RcNtrC protein binds to tandem sites greater than 90bps upstream of at least five RcNtrC-dependent promoters, establishing the "remote" nature of this *R. capsulatus* system (Bowman, Kranz, 1998). However, ATP binding, but not hydrolysis, to RcNtrC is sufficient for activation. Surprisingly, the σ^{70}RNAP is activated at these promoters (e.g., *nifA1).* Thus, considering the principles that have emerged from the *E.coli* promoter database compilation, the RcNtrC system represents a third type of activator in *Rhodobacter.* The first type is represented by RegA (proximal binding at σ^{70}/RNAP promoters) and the second is the traditional EBP/σ^{54}/RNAP types represented by the NifA EBP (Cullen *et al.,* 1994).

3. Alpha Proteobacterial Promoters: Why isn't the *E.coli* σ^{70}/RNAP Activated at the *nifA1* and *puc* and *puf* Promoters by their Respective Activators?

The alpha proteobacterial lineage is predicted to have diverged from the gamma group over 600 million years ago. Thus, new regulatory systems and even properties of the RNAPs have had ample time to evolve independently in the alpha and gamma lineages. Today's alpha proteobacterial species possess high GC contents in their genomic DNA, typically greater than 65%, another consideration when evaluating promoters. Selected reports on promoters from some of the better-studied alpha proteobacteria, *Caulobacter, Agrobacterium,* rhizobia, and *Rhodobacter* are reviewed here.

Caulobacter cresentus generates two different cell types, swarmer and stalk cells, prior to cell division and is a model organism for cell differentiation. Early investigations on *C. cresentus* and other alpha proteobacteria attempted to clone genes by complementing *E. coli* auxotrophs. Malakooti *et al.* (1995) suggest these studies were often unsuccessful because the promoters were not recognized and/or activators were not present in the heterologous host. Recent *in vitro* studies show that the purified RNAP from C. *cresentus* can transcribe *E.coli* promoters that have good -35 and -10 regions (e.g., *lacUV5, neo)* and *C. cresentus* housekeeping promoters *(pleC, rsaA)* (Wu *et al.,* 1997). S1 nuclease mapping was used to determine the start sites and thus promoters *pleC, rsaA* and other housekeeping genes, and *lacZ* fusion analysis combined with promoter mutagenesis determined promoter strength (Malakooti *et al.,* 1995). While the ideal *C. cresentus* -35 is similar to the *E. coli* consensus (TTGACA), a more GC-rich and less specific -10 region also was suggested (compared to the *E.coli* TATAAT consensus). An important accompanying paper (Malakooti, Ely, 1995) showed that the *C. cresentus rpoD* gene encoding σ^{70} was able to complement an *E. coli rpoD* temperature-sensitive mutant. Additionally, the *C. cresentus rpoD* gene increased the transcription of the *pleC-lacZ* fusion in *E. coli* approximately four-fold. This result suggested that the σ^{70} subunit in part facilitates the increased recognition/transcription of the C. *cresentus* genes.

Agrobacterium tumefaciens is the causative organism of crown gall disease and contains the major vector (Ti-plasmid) for transferring genes into plants. S1 nuclease mapping determined the start site and promoters for six genes encoded by the Ti-plasmid, *virA, B, C, D, E,* and *G* (Das *et al.,* 1986). All but *virA* is activated by the response regulator VirG and thus the -35 regions are likely not ideal. Very recently, an inability to reconstitute the VirG activation of *vir* promoters in *E.coli* led to a search for other factors involved (Lohrke *et al.,* 1999). This search showed that VirG could activate a *virB-lacZ* fusion in *E.coli* only when the *A. tumefaciens* RNAP α subunit was expressed. *In vitro* reconstitution of a hybrid RNAP containing *E.coli* RNAP β, β',σ subunits and the *A. tumefaciens* α subunit confirmed that VirG interacts with the *A. tumefaciens* but not *E. coli* α subunit. Thus, this interaction has co-evolved and *E. coli* has lost determinants of the RNAP interaction. The *virB* promoter has the -10 sequence GATAAT, very similar to the *E.coli* consensus (TATAAT) which is likely another reason that this experiment was successful. It is worth noting that some of the other *vir* genes possess less characteristic -10 hexamers of higher GC content (Das *et al.,* 1986).

Rhizobia are responsible for the agriculturally important symbioses of legumes. An early *in vitro* study indicated that the *Rhizobium meliloti* RNAP but not the *E. coli* RNAP could transcribe the *nodD* promoter (Fisher *et al.,* 1987). On the other hand, the *R. meliloti* RNAP could appropriately initiate transcription of an ideal *E. coli* promoter. Also, the *fixK* promoter could be activated by FixJ using the σ^{70} RNAP from *E. coli,* suggesting that some promoters are recognized by the *E.coli* holoenzyme (Batut *et al.,* 1991). Recently, the purified RNAPs from *Bradyrhizobiumjaponicum* and *E. coli* were used to analyze *in vitro* transcription of the *B. japonicum rrn* promoter and some mutant variants (Beck *et al.,* 1997). Their main conclusion is that the specificities appear to be very similar, with ideal -35 and -10 regions close to the *E. coli* consensus hexamers. A clear determination of whether the rhizobia have a more general -10 requirement remains open.

R. capsulatus and *Rhodobacter sphaeroides* have been the primary models for gene regulation in phototrophs. A study by Karls *et al.* on the *R. sphaeroides* and *E. coli* RNAPs suggested that *R. sphaeroides rrn* promoters were only recognized by the *R. sphaeroides* RNAP, although *E. coli* promoters (eg. *lacUV5)* were recognized by both (Karls *et al.,* 1993). They could not rule out that another sigma factor in the *R. sphaeroides* preparations was responsible. Our group has defined the housekeeping promoters in *R. capsulatus* by a combination of *in vivo* primer extension studies, *in vitro* transcription analysis of selected genes and mutation of the -35 and -10 regions of the *nifA1* promoter (Cullen *et al.,* 1997). Transcription of these promoters with the purified *E. coli* and *R. capsulatus* RNAPs confirmed a σ^{70} requirement (Bowman *et al.,* 1999; Bowman, Kranz, 1998). The *R. capsulatus* RNAP can efficiently recognize the *E. coli* -35/-10 consensus promoters. The -35 region consensus is similar to the *E. coli* -35 hexamer (TTGACA). Importantly, the *R. capsulatus* σ^{70} RNAP can transcribe genes where the -10 is higher in GC than the *E.coli* consensus, suggesting a more promiscuous σ^{70} subunit interaction to melt the -10 region in *R. capsulatus.*

If co-evolution of activator:RNAP subunit interactions presents one problem for activation in heterologous *E. coli,* does the -10 region of some promoters from the alpha proteobactia also present a second barrier (e.g., evolved towards higher GC composition)? None of the *R. capsulatus* activated promoters (*puc, puf, nifA1*) function with the *E. coli* RNAP. As with the *A. tumefaciens* **VirG/α** subunit interaction, we believe that the sites of interaction on the *R. capsulatus* RNAP have co-evolved with these activators. A second potential barrier resides in the promiscuity of the *R. capsulatus* σ^{70} subunit compared to the *E. coli* subunit, e.g., the *nifA1* -10 region contains the hexamer TATGGT. Using the recombinant *R. capsulatus* σ^{70} subunit with the *E.coli* core at 37°C, we showed that the *E.coli* hybrid RNAP can now efficiently melt/transcribe this -10 region (Sloan, Kranz, unpublished). These studies used mutant templates where the -35 region was changed to make transcription independent of RcNtrC both *in vitro* and *in vivo,* i.e., constitutive in *R. capsulatus.* A major conclusion is that the *R. capsulatus* σ^{70} subunit and RNAP core have evolved to efficiently transcribe either typical TATAAT or higher GC -10 regions.

We are moving towards the ability to predict which *R. capsulatus* RNAP subunit(s) will be necessary to reconstitute *Rhodobacter* promoters and activation systems in *E.coli.* Possibly the same rules will apply to other alpha genera. An emerging theme in alpha proteobacterial transcription is the more general recognition determinants of the -10 region of promoters. Theoretically, this could mean that the ideal -35 hexamers (TTGACA) must be less frequent throughout the genome and that activation systems must be prevalent so that aberrant transcription initiation does not take place. One of the ramifications of evolving a more promiscuous housekeeping RNAP is that predicting promoters *a priori* becomes less feasible. Even if *in vivo* transcription start sites were determined on a large database of promoters, the establishment of a -10 consensus might not be possible. On the other hand, with the completion of the genomic sequencing of a few alpha proteobacteria over the next couple years, it would be worth generating such a transcription start site database on one of them to determine if such a consensus emerges. If so, it might also be applicable to many of the alpha genera.

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REGULATION OF NITROGEN FIXATION AND GLUTAMINE SYNTHETASE IN *HERBASPIRILLUM SEROPEDICAE*

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1. Introduction

Herbaspirillum seropedicae is a nitrogen-fixing bacterium which associates endophytically with several plants and which fixes nitrogen under microaerophilic conditions in the absence of a fixed nitrogen source (Baldani et al., 1986, Boddey et al., 1995). Initially classified as an *Azospirillum* species, it is now placed in the β-subdivision of the Proteobacteria (Young, 1992). This subdivision, which is the least studied genetically of nitrogen-fixing families, includes species of *Rhodocyclus, Alcaligenes, Derxia* and *Azoarcus*. Recent evidence shows that *H. seropedicae* can contribute significantly to the associated plant nitrogen budget (Boddey et al., 1995).

In free-living and associative diazotrophs nitrogen fixation is repressed by environmental ammonium, a preferential nitrogen source, and oxygen, which inactivates nitrogenase. In all studied nitrogen-fixing Proteobacteria the NifA protein is a specific activator of the expression of *nif* gene promoters by interaction with σ^N (σ^{54},RpoN)-RNA polymerase, which recognises promoters with the dinucleotides GG and GC conserved at positions -25/-24 and -12/-13, respectively. The regulation of expression of *nifA* varies considerably, however, differing in the involvement of ammonium or oxygen and the nature of the regulatory protein. Ammonium and oxygen can also regulate NifA activity either directly or through the NifL protein.

Arsène et al. (1996) and Souza et al. (1999) have recently shown that in *Azospirillum brasilense* and in *H. seropedicae,* members of the alpha and beta subdivisions of the Proteobacteria, respectively, the N-terminal domain of NifA mediates nitrogen control. Apparently this does not occur in rhizobia, which export ammonium to the host plant, and lack nitrogen regulation at the level of *nif* expression. In *Klebsiella pneumoniae, Enterobacter agglomerans* and *Azotobacter vinelandii,* members of the gamma subdivision of the proteobacteria, NifA itself is neither ammonium- nor oxygen-sensitive, but its activity is controlled by the NifL protein, which under high ammonium or oxygen concentrations binds NifA, inhibiting transcription activation. Rhizobial NifA proteins are redox-sensitive, but this appears to be a property not of the regulatory domain, but of a characteristic C-terminal extension of the central domain that includes conserved cysteine residues, a feature also present in the *A. brasilense* and *H. seropedicae* proteins. The transcriptional activity of rhizobial NifA proteins is inhibited by the presence of chelating agents in the medium, an effect which can be overcome by adding excess Fe, prompting the speculation that oxygen control of NifA activity is mediated by the redox state of Fe bound by the conserved cysteine residues.

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2. The *nif* genes in *H. seropedicae*

The *nif* genes in *H. seropedicae* are grouped in at least two independent clusters. One contains the *nifA* and *nifB* genes organized in two distinct operons. The second contains the majority of the *nif* genes of *H. seropedicae* and comprises *nifHDKENXorf1orf2orf3 nifQmodABC*. Mutation studies indicate that *nifN* and *nifB* are essential for nitrogenase activity in *H. seropedicae* whereas mutations in *nifX* and *orf1* did not affect nitrogen fixation. Sequence analysis suggested that *orf1* probably codes for a ferredoxin-like protein homologous to the *orf4* from *Rhodobacter capsulatus* (Moreno-Vivian et al, 1999) but *orf2* and *orf3* had no significant similarity to any protein in the database. Sequence analysis and transcriptional studies with *lacZ* fusions showed that *nifHDKENXorf1orf2orf3* comprises a single operon expressed in the absence of ammonia and low oxygen concentration, under control of the NifA- and RpoN-dependent *nifH* promoter (Machado et al., 1997, Klassen et al., submitted). The *nifB* promoter is also activated by NifA *in vivo* and it has been shown that NifA and RpoN proteins bind to it *in vitro* and *in vivo* (Rego et al., submitted). The *nifQ* and *modA* genes were essential for nitrogen fixation only under low molybdenum conditions and the *modA* gene was essential for nitrate-dependent growth. No promoter activity was found in the *nifQ-modA* intergenic region suggesting that the *nifQmodABC* genes are organized in a single operon and probably these genes are expressed from the *nifQ* promoter.

3. Nitrogen metabolism control in *H. seropedicae*

The *H. seropedicae ntrC* null mutant (DCP286A) was obtained by transposon Tn5-B20 insertion. Strain DCP286A was Nif^- and failed to grow on nitrate as the sole N source but grew as well as the wild type on several other nitrogen source. Both phenotypes (nitrate growth and nitrogenase activity) were complemented by plasmid pKRTl, which carries the wild type *glnAntrBC* operon.

Analysis of the *nifA* promoter sequence (Souza et al., 1991) and the Nif^- phenotype of the *ntrC* mutant indicated that *nifA* expression in *H. seropedicae* was NtrC-dependent. To confirm this suggestion a *nifA::lacZ* fusion was introduced into *H. seropedicae* strains SMR1 (wild type) and DCP286A (*ntrC*). The *nifA* expression in the wild type strain SMR1 increased significantly under nitrogen-fixing conditions whereas in the *ntrC* mutant no activation was detected. Furthermore, plasmid pEMS135, which constitutively expresses *nifA* from the *lacZ* promoter, complemented strain DCP286A for nitrogenase activity.

4. Expression and adenylylation of glutamine synthetase

The β-galactosidase activity of a *glnA::lacZ* fusion and the total level of GS (total transferase activity) in the wild type SMR1 increased 2-3 fold when grown under nitrogen-limitation whereas GS activity in the *ntrC* mutant was unaffected by a change in the nitrogen status, indicating that NtrC activates the expression of *glnAntrBC* in response to nitrogen. The GS biosynthetic activity, which reflects the non-adenylylated (active GS) fraction, was low in the *ntrC* mutant regardless of the N levels. An increase occurred in the biosynthetic activity of GS when the wild-type strain was shifted from high to low ammonium, regardless of the presence of protein synthesis inhibitors, tetracycline or chloramphenicol, whereas no increase was observed in the *ntrC* mutant. Addition of 20 mmol/L NH_4Cl caused a decrease of GS levels, restoring the initial biosynthetic activity in the wild type but, again, no change occurred in the mutant, indicating a major role for NtrC in adenylylation/deadenylylation of GS. Since *glnB* expression is constitutive in *H. seropedicae* and a *glnB* mutant has a normal GS adenylylation pattern (Benelli et al 1997), the data suggest the presence of a second, NtrC-dependent, signal transducer in *H. seropedicae* perhaps corresponding to the GlnK protein of enteric bacteria (van Heeswijk et al., 1996; Atkinson & Ninfa, 1998). These results suggest that NtrC is involved both in the regulation of GS synthesis and activity control.

5. *H. seropedicae nifA* promoter regulation

The promoter region of the *nifA* gene of *H. seropedicae* contains sequences homologous to a -24/-12 type promoter, together with NifA- and NtrC-binding sites (Souza et al., 1991), suggesting participation of these proteins in the expression of the *nifA* gene. The activity of the NifA protein from *H. seropedicae* is controlled by ammonium ions and oxygen (Souza et al., 1999) and apparently requires a functional *glnB*-like gene (Benelli et al., 1997).

A *nifA* RNA start under derepressing conditions was identified at 12 bp upstream from the putative -24/-12 promoter element *(nifAp).* Mutation at either positions –24 or –13 produced an inactive promoter, indicating that this sequence is indeed the functional promoter of the *nifA* gene of *H. seropedicae. In vivo* footprinting in *H. seropedicae* of this promoter region showed that the G residues at position –26 and –9 are hypermethylated towards DMS and that the region from –10 to +10 is partially melted under nitrogen-fixing conditions confirming that this is the active *nifA* promoter.

In the wild type *H. seropedicae* strain SMR1 pRW1 (native *nifA::lacZ)* the β-galactosidase activity was 4-6 fold higher in the absence than in the presence of ammonium ions, whereas oxygen caused only a 20-30% drop in *nifA* expression. The presence of a putative NtrC-binding sequence and a -24/-12 type promoter (Souza et al., 1991) together with the results in *E. coli* suggested that NH_4^+ levels regulate *nifA* expression in *H. seropedicae* in a NtrC-, σ^N-dependent fashion. The native *nifA* promoter failed to derepress in the *ntrC*$^-$ strain DCP286A (Persuhn et al. submitted) confirming the requirement of NtrC as the primary activator.

6. NifA-dependent activation

The presence of a NifA-binding site (position -130) upstream from the –24/–12 promoter sequence suggested auto-activation (Souza et al., 1991). Under low oxygen and absence of ammonium the *nifA* promoter activity increased 50% in the wild type strain. The expression in the *nifA*$^-$ strain SMR54 of the native *nifA* promoter was regulated by ammonium but not affected by oxygen. In the absence of ammonium, the level of activity in the *nifA* mutant under 1.5% oxygen compares with that under air (20% oxygen) in both the wild type and *nifA* mutant. Moreover, deletion of the NifA-UAS reduced promoter activation under low oxygen in the wild type strain to a level similar to that in the presence of high oxygen. Since *H. seropedicae* NifA protein is oxygen-sensitive (Souza et al., 1999) these results suggest that full activation of the *nifA* promoter requires NifA and that auto-activation occurs through interaction with its putative UAS. Indeed, *in vivo* DMS footprinting of the NifA UAS of the *nifA* promoter indicated NifA binding under nitrogen-fixing conditions. Both activators NtrC and NifA seem to activate expression of the –24/–12promoter. When the conserved guanines at -24 and -13 positions are mutated to thymines, the promoter activity is abolished in both *E. coli* and *H. seropedicae.*

7. IHF-binding site inhibits NifA auto-activation

Upstream from the NifA UAS, an IHF-binding site was identified (Souza et al., 1991). This sequence was deleted (position -200 to -160) and the resulting *lacZ* fusion (pRW7) introduced into *H. seropedicae.* First, in the absence of ammonium ions and under 1.5% oxygen **β-galactosidase** activity in the wild type strain-carrying the IHF-deleted fusion was 2 fold higher than that with the native promoter. In the *nifA* strain SMR54, however, this effect was not observed. These results indicate that the IHF-binding sequence upstream from the NifA UAS inhibits NifA-dependent transcription.

8. *In vitro* studies of *nifA* expression

To determine the involvement of these regulatory elements in the promoter regulation *in vitro* transcription activation assays were performed in a system with purified proteins where the *nifA* promoter was cloned into the transcription vector pTE103. The DNA bending protein IHF stimulates activation of the *H. seropedicae nifA* promoter by NtrC. In marked contrast activation by NifA is greatly reduced by IHF. Additionally, in the presence of GTP high levels of NifA appear to limit NtrC-dependent activation and NifA can inhibit its own transcription. This inhibition is dependent on the presence of IHF. Therefore IHF acts positively and negatively at the *nifA* promoter to restrict transcription activation to a single activator and one signal transduction pathway.

DNA-footprinting experiments showed that the potential enhancers for the nitrogen control activators NtrC and NifA and binding-sites for IHF and **σ^N-holoenzyme** functioned for protein binding. No evidence for competition between NifA and IHF was observed, suggesting that IHF binding at the *nifA* promoter interferes with the proper interaction between the NifA bound protein and the **σ^N-RNA** polymerase but not with NifA binding.

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ANALYSIS OF BACTERIAL GENE EXPRESSION DURING THE LATE STAGES OF THE INTERACTION BETWEEN *RHIZOBIUM ETLI* CNPAF512 AND *PHASEOLUS VULGARIS.*

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Rhizobium etli CNPAF512 is the nodulating symbiont of the common bean plant, *Phaseolus vulgaris.* We have identified three different regulatory cascades of nitrogen fixation genes. The first cascade is dependent on the transcriptional regulator NifA and controls the expression of both *nifHDK* operons, the third *nifH* gene, which is not linked to other *nif* genes, and the production of the black pigment melanin (Michiels, Vanderleyden 1993, Michiels et al 1994; Valderama et al 1996). Although the *R. etli nifA* gene is transcribed under aerobic and microaerobic conditions, NifA-dependent gene activation is only operative at low oxygen tensions (Michiels et al 1994). Target gene activation by the NifA protein requires the activity of the alternative sigma factor RpoN or σ^{54}, which recognizes a conserved sequence motif centered at -24 and -12 nucleotides from the transcriptional start. We have recently identified two *rpoN* genes in *R. etli,* called *rpoN1* and *rpoN2* (Michiels et al 1998a, 1998b). Under free-living conditions, *rpoN1* controls growth on C_4-dicarboxylic acids and on several nitrogen sources. In addition, inactivation of *rpoN1* abolishes microaerobic expression of *nifH* and the production of the black pigment melanin in free-living conditions (Michiels et al 1998a). The second *rpoN* gene, *rpoN2,* is cotranscribed with a short open reading frame, *orf180.* The *rpoN2* gene has no obvious function during free-living growth whereas it is essential during symbiosis (Michiels et al 1998b). Plants nodulated by a *R. etli rpoN2* mutant strain have a reduction in nitrogen fixation by approximately 90% compared to the wild type. Expression of the *R. etli rpoNl* gene is negatively autoregulated while transcription activation of the *orf180rpoN2* operon strictly requires the NifA protein (Michiels et al 1998a, 1998b). In free-living microaerobic cultures, *rpoN2* expression is controlled by the RpoNl protein. In contrast, in bacteroids, the *rpoN2* gene is transcribed independently of the *rpoN1* gene, suggesting the existence of an alternative symbiosis-specific mechanism of transcription activation. Recent evidence suggests the presence of a third *rpoN-like* gene in *R. etli* (B. Dombrecht, unpublished).

The second cascade is controlled by the *R. etli fixLJ* genes (D'hooghe et al 1995). Unlike the FixL protein from *Sinorhizobium meliloti* (Gilles-Gonzales et al 1991), *R. etli* FixL is not a haemprotein (D'hooghe et al 1998). The physiological signal sensed by *R. etli* FixL is presently unknown. Recent evidence suggests that the *R. etli fixLJ* operon might be involved in the symbiotic transcriptional regulation of an ABC-type of transporter (J. Michiels, unpublished).

We have recently identified a third regulator of nitrogen fixation genes in *R. etli,* an *fnrN-like* gene (J. Michiels, unpublished). The FnrN protein possesses a conserved arrangement of cysteine residues which is characteristic of redox-sensitive FnrN-like proteins (Spiro 1994). The presence of an active FnrN protein is essential for an efficient symbiosis with *P. vulgaris.* This protein is required for the expression of the *R. etli fixN* and *fixG* genes. We have previously identified an arginine deiminase pathway in *R. etli* which is active in bacteroids (D'hooghe et al 1997). The corresponding *arcABC* genes code for an arginine deiminase, a catabolic ornithine

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carbamoyl-transferase (cOTCase) and a carbamate kinase. These enzymes are involved in the degradation of arginine with the generation of ATP. Bean nodules formed by an *R. etli arcA* mutant strain have a reduced nitrogen fixation activity. OTCase activity in bacteroids is active at a different pH optimum (pH 6.8) as compared to the OTCase activity in free-living cells, suggesting the presence of two enzymes with different characteristics and different expression patterns (D'hooghe et al 1997). The expression of the *arcA* gene was demonstrated to be dependent on the identified *fnrN* gene (J. Michiels, unpublished).

To isolate new symbiotic genes expressed during the late stages of the interaction and to identify new signals operating at this stage, we designed a strategy based on the differential expression of a *R. etli* mutant library tagged with a *gusA*-promoter-probe transposon (see figure, Xi et al 1999). GusA activity of these mutants was tested under conditions of low oxygen and/or in the presence of bean nodule extracts. Mutants displaying induced GusA activity under these conditions were further characterised. Using this approach, mutants carrying insertions in nodulation and nitrogen fixation genes as well as genes coding for other functions were isolated (C-W Xi, unpublished).

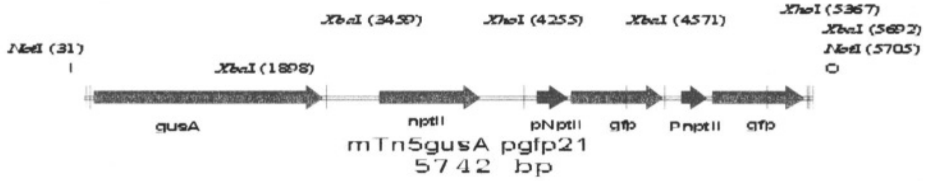

Figure: Schematic representation of the mini-Tn*5* transposon-derivative mTn*5gusA-pgfp21*, used to construct an *R. etli* mutant expression library. The transposon contains a promoterless *gusA* gene containing three translational stops upstream from the gene and generates transcriptional *gusA* fusions with the target. The two *gfp* genes are under the control of the *npt*II promoter and confer constitutive GFP fluorescence to the bacterium (Xi et al. 1999).

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Acknowledgements

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FUNCTIONAL ANALYSIS OF THE *BRADYRHIZOBIUM JAPONICUM* REGSR TWO-COMPONENT REGULATORY SYSTEM

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1. Introduction

Microaerobic expression of many genes required for nitrogen fixation in *Bradyrhizobium japonicum* depends on the activator protein NifA encoded in the *fixRnifA* operon, which is preceded by two disparately regulated promoters *(fixRpl, fixRp2).* In aerobic conditions, low-level expression from *flxRp2* depends on the response regulator RegR (Bauer et al. 1998), whereas NifA enhances expression under low-oxygen conditions via activation of *fixRpl* (Thöny et al. 1989). RegR was found to be encoded downstream of *regS.* The deduced amino acid sequences suggested that RegR and RegS represent a two-component regulatory system (Bauer et al. 1998). Here we report a more detailed analysis of RegR and RegS with regard to their function *in vitro* and *in vivo.* Furthermore, we present evidence that the RegSR proteins are functionally similar to the RegBA proteins, which are involved in the control of photosynthesis gene expression in *Rhodobacter capsulatus.*

2. Results

For biochemical characterization of RegR and the putative sensor kinase RegS, we purified His-tagged derivatives of RegR and of a soluble, cytoplasmic C-terminal domain of RegS (RegSc). Purified RegSc showed rapid, progressive autophosphorylation when incubated with radioactive $[\gamma\text{-}^{32}\mathrm{P}]\mathrm{ATP}$. After addition of RegR the phosphate group was transferred from RegSc~P to RegR. To investigate if RegS~P has phosphatase activity, we incubated pre-phosphorylated RegR with RegSc~P The amount of radiolabel in RegR~P decreased progressively, resulting in the loss of more than 90% radioactivity after 162 min. Amino acid sequence alignments suggested that H219 of RegS and D63 of RegR are the phosphorylated residues. To test this inference, we have constructed by site-directed mutagenesis the mutant proteins RegSc-H219D and RegR-D63N, which both showed no (auto)phosphorylation activity.

The DNA-binding activity of purified RegR was tested in gel retardation experiments using an oligonucleotide that spans the upstream region (UAS) of the *fixRnifA* promoter. Binding of purified RegR retarded the electrophoretic mobility of the *fixR* UAS oligonucleotide. UAS binding was dependent on phosphorylation of RegR, as only very weak complex formation was observed with dephosphorylated RegR or with RegR-D63N. Quantitative analysis revealed that DNA-binding of RegR was enhanced at least eight-fold by phosphorylation. To more precisely define the DNA binding specificity of RegR we performed band-shift experiments with 46 different oligonucleotides comprising various point or deletion mutations in *the fixR* UAS and with an *in vitro* binding-site selection assay (SELEX). We identified 12 critical nucleotides within a 17 bp minimal RegR binding site centered at position –64 upstream of the *fixRnifA* transcriptional start site. A 5 bp imperfect inverted repeat located within this region is required but not sufficient for RegR binding.

Sequence comparisons indicated a high overall similarity of RegR to RegA from *Rhodobacter capsulatus,* which reaches 100% identity in the putative helix-turn-helix motifs of these proteins. Comparison of the RegR binding site with the three known for RegA*, a constitutively active mutant variant of RegA (Du et al. 1998, 1999), revealed substantial similarity. In fact, we were able to demonstrate binding of RegR to *R. capsulatus puc* and *puf* promoter fragments, which represent genuine targets for RegA binding. Conversely, the *R. capsulatus regA* gene could at least partially

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complement a *B. japonicum regR* deletion mutant as indicated by the restoration of aerobic ***fixR*′-′*lacZ*** expression and of the symbiotic properties.

The role of RegR phosphorylation on gene activation *in vivo* was analyzed by chromosomal integration of the *regR** gene, coding for RegR-D63N, into a *regR* deletion mutant, which harbored a ***fixR*′-′*lacZ*** fusion. In aerobic conditions, **β-galactosidase** activity derived from the reporter was reduced 20-fold in the *regR**-complemented mutant as compared with the wild type. Surprisingly, nodules induced by the *regR**-complemented strain exhibited 67% of wild-type Fix activity.

3. Discussion

We have shown that the RegR and **$\mathrm{RegS_C}$** proteins exhibit the *in vitro* properties that are typical for two-component regulatory proteins, i. e., autophosphorylation, phosphoryl transfer and specific DNA binding (Emmerich et al. 1999). Previously, it was shown that *regS* is dispensable for symbiosis (Bauer et al. 1998). The fact that phosphorylation of RegR is required for *in vitro* DNA-binding and aerobic expression of the *fixRnifA* operon could mean that RegR is phosphorylated *in vivo* via cross talk by another, functionally related kinase or by a low-molecular-weight phosphodonor. Phosphorylation of RegR does not seem to be absolutely required for symbiosis as indicated by the **Fix^+** phenotype of the *regR*-containing* mutant. It appears as if expression of minimal amounts of NifA is sufficient for *fixRnifA* autoactivation and, in turn, for expression of symbiotic genes. The facts that RegR is able to bind to the promoter region of *R. capsulatus* photosynthesis genes *(puc, puf)* and that *regA* can correct the symbiotic defect of a *B. japonicum regR* deletion mutant further substantiate the earlier notion about a regulatory link between nitrogen fixation, photosynthesis and autotrophy (Joshi and Tabita 1996). Interestingly, we recently obtained evidence that the regulatory scope of RegR in *B. japonicum* extends to the process of **CO_2** fixation as indicated by the RegR-dependent expression of a ***cbbL*′-′*lacZ*** fusion.

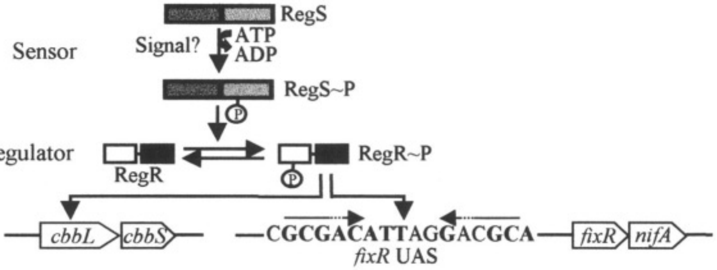

Fig. 1 The emerging role of the *B. japonicum* two-component regulatory system RegSR in CO_2 and N_2 fixation. Currently known members of the RegR regulon are the *cbbLS* genes encoding ribulose-1,5-bisphosphate carboxylase (rubisco) and the *fixRnifA* operon. Note that it is presently unknown whether RegR controls *cbbLS* expression directly or indirectly. Essential nucleotides in the minimal RegR binding site are indicated in bold face. The horizontal arrows above the *fixR* UAS mark an imperfect inverted repeat.

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ROLE OF *HERBASPIRILLUM SEROPEDICAE* NIFA DOMAINS ON THE EXPRESSION OF *nif* GENES.

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H. seropedicae NifA is a transcriptional activator protein of *nif* genes. This protein comprises three domains: the C-terminal domain contains a helix-turn-helix motif that is responsible for binding to specific activator-binding sites (UAS) of *nif* gene promoters. The central domain interacts with the σ^N subunit of the RNA polymerase to catalyze the open complex formation (Kustu et al., 1989). The N-terminal domain seems to have a regulatory function, responsible for the sensitivity of NifA to NH_4^+ (Souza et al., 1991; Souza et al., 1999; Monteiro et al., 1999)

The N-truncated NifA protein was over-expressed as a fusion protein with a His-tag after induction with IPTG. The majority of the expressed fusion protein was insoluble. Attempts to obtain a soluble protein by changing the conditions of induction and using denaturing agents were unsuccessful. However, when lactose was used as the inducing agent instead of IPTG, up to 40% of the over-expressed protein was soluble. A time course of induction clearly indicated a lower induction rate by lactose than by IPTG. We suggest that the slower induction rate may allow correct folding of the protein. This soluble His-tagged N-truncated NifA was purified on a HiTrap-Chelating-Ni^{2+} affinity column eluted with imidazole, yielding a high purity (97%) fraction (Monteiro et al.,1999).

Band shift assays, using 30-fold molar excess of protein to DNA showed that the N-truncated NifA bound specifically to a [^{32}P]-labelled fragment of the *nifB* promoter region: the protein-DNA complex was stable in the presence of 150-fold excess of calf thymus DNA while the radioactive label was titrated out by excess unlabelled *nifB* promoter DNA. DMS footprinting verified that the N-truncated NifA bound to the two upstream NifA UAS of the *nifB* promoter: the guanine residues of the TGT motifs of both NifA-binding sites were protected from DMS-dependent methylation by the N-truncated NifA protein. This result showed that the N-truncated NifA binds to both UAS present in the *nifB* promoter region, indicating that the N-truncated NifA was capable of recognizing and binding both sites. These DNA-protein interactions were performed under air. Since *in vivo* experiments have shown that the *H. seropedicae* NifA is oxygen-sensitive (Souza et al., 1999; Monteiro et al.,1999), the observed DNA binding might have been due to a transcriptionally inactive protein, or alternatively, that oxygen damage was partial and only a functionally active population of the N-truncated NifA bound to the *nifB* promoter. DNA binding of a functionally inactive protein would be consistent with the hypothesis that the C-terminal domain binds DNA and the oxygen sensitivity is associated with the cysteine motif located in the central domain and ID linker and may not interfere with the DNA-binding activity.

The N-truncated NifA was tested *in vivo* for the capacity to activate the expression of the *K. pneumoniae nifH* promoter (*nifH::lacZ* fusion). *E. coli* strain JM109(λDE3) transformants hosting plasmids pET28-a (vector), pRAM2 (His-tagged N-truncated NifA) or pRT22 (*nifH::lacZ*) were analysed alone or in combinations for β-galactosidase activity after induction in the presence or absence of oxygen or NH_4Cl. The N-truncated NifA protein was able to activate the transcription

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from the *nifH* promoter in *E. coli* in the absence of oxygen, independent of the NH_4Cl levels. However, when plasmid pRAM4, expressing the N-terminal domain of the NifA protein *in trans* was also present in the system, the activity of the central+C-terminal domain was inhibited 70% in the presence of 20mM NH_4Cl. Both proteins were expressed from the T7 promoter and SDS-PAGE showed that their levels of expression were similar, the central+C-terminal domains represented 12% of the total protein and the N-terminal domain 10%. This situation resembles that in *H. seropedicae* or *Azospihllum brasilense* where the activity of the native NifA is controlled by the levels of ammonium (Souza et al.,1999; Arsene et al., 1996). The inhibition of NifA in the presence of ammonium may be due to the binding of the N-terminal to central+C-terminal domains. When native *H. seropedicae* NifA was introduced into *E. coli* it was inactive, indicating that the presence of the N-terminal domain made it permanently inhibited (Souza et al., 1999; Monteiro et al.,1999). The isolated N-terminal domain seems to have acquired the ability to recognize a signal molecule in *E. coli,* presumably as a consequence of the protein truncation.

To determine whether the effect of the N-terminal domain was dependent on protein concentration, different promoters were used *(lac* and *tac* promoters) to achieve expression. When the central+C-terminal domain was expressed from the *lac* promoter (pRAM7) in *E. coli* YMC10 cells, β-galactosidase activity was detected as a result of *nifH::lacZ* activation. Co-expression of the N-terminal domain from the stronger *tac* promoter (pRAM9) led to inhibition of transcription activation by the central+C-terminal domains in the presence or absence of ammonium. These results confirm that the N-terminal domain can interact with the central+C-terminal domains to inhibit transcriptional activity dependent on the presence of ammonia only when the domains are equally expressed.

The interaction between the N-terminal domain and the central+C-terminal domains was tested *in vitro* by purifying the over-produced His-tagged N-terminal domain and testing its effect in a band-shift assay. When present at a four-fold molar excess concentration, the N-terminal domain inhibited the binding of the central+C-terminal domains to the *Rhizobium meliloti nifH* promoter. This result suggests that the N-terminal domain inhibits NifA activity *in vivo* by inducing a conformational change in NifA to prevent binding to the NifA UAS'.

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CHARACTERIZATION OF AN *AZOSPIRILLUM BRASILENSE* TN5 MUTANT WITH ENHANCED NITROGEN FIXATION.

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1. Introduction

In the diazotrophic bacterium *Azospirillum brasilense* the expression of nitrogen fixation genes is regulated in response to changes in oxygen concentration and nitrogenous compounds. An *A. brasilense* Tn5 mutant previously identified with enhanced expression of the nitrogenase structural genes was further analyzed. This mutant is also less sensitive to high oxygen concentration with respect to *nifH* expression. A 12 kb *Eco*RI DNA fragment containing the Tn5 insertion was cloned and the nucleotide sequence of the Tn5 flanking regions was established. Analysis of the nucleotide sequence of the Tn5 flanking regions led to the identification of two open reading frames, encoding polypeptides of 281 and 278 amino acids, named ORF281 and AtrAb respectively. A broad host range cosmid containing the *A. brasilense* ORF281 gene was shown to restore the *A. brasilense* mutant nitrogen fixation activity to wild-type level, indicating that ORF281 may be responsible for the altered phenotype. The involvement of ORF281 on the regulation of the nitrogenase structural gene expression is discussed.

2. Materials and methods

General cloning techniques were carried out as described by Sambrook et al., (1989). Growth of *A. brasilense* on different nitrogen source was tested on NFbHP (Machado et al., 1991) supplemented with 5mM glutamine, arginine, histidine, serine, lysine or glutamate.

β-glucuronidase activity of the culture was determined quantitatively using the substrate ***p*-nitrophenyl-β-D-glucuronide** as described previously (Jefferson, 1987). The acetylene reduction assays on whole cells derepressed for nitrogenase were performed as previosly described (Vande Broek et al., 1996). Nitrogenase activity was measured by the acetylene reduction assay as described previously (Araujo et al., 1988).

3. Results and Discussion

A. brasilense ORF281 is similar to *Bradyrhizobium japonicum* ORF277, *Paracoccus denitrificans* ORF278 and *Rhodobacter capsulatus* ORF277 Up to now, no specific function could be assigned to ORF277 and ORF278. Expression *of* a *nifH-gusA* fusion in the *A.brasilense* ORF281-mutant strain revealed tenfold-higher *nifH* expression than the wild-type strain and at oxygen levels not permissive for nitrogen fixation. These results provide evidence concerning the possible involvement of the ORF281 gene product on *nifH* promoter expression during nitrogen fixation in *A. brasilense* (Revers et al.,1999). By computer analises of the ORF281 we found at C-terminus, the USP motif. This motif is typical of the new family of universal stress proteins (Makarova et al., 1999). Upstream ORF281 gene an ORF of 834 nucleotides called AtrAb, that potentially codes for a polypeptide of 278 amino acids, was found (Fig. 1). AtrAb shares a high degree of homology with the LysR family of autoregulatory transcriptional regulators (Schell. 1993) Alignment of the AtrAb aminoacid sequence and proteins of the LvsR

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family revealed that the highest similarity scores are found at the N-terminus region containing a DNA- binding helix-turn-helix motif AtrAb may be considered as a transcriptional regulatory protein based on the common features related to the LysR family as: (i) presence of the *h-t-h* motif at the amino terminus, (ii) divergent transcription in relation to ORF281, (iii) the low ratio content of Lys codons to Arg codons . Typical growth curves for the wild-type and mutant strain are shown on liquid minimal medium supplemented with NH_4Cl, glutamate or arginine as nitrogen source. The growth rate of the mutant decreased drastically when glutamate was used as nitrogen source. This unexpected behavior of the mutant was further analyzed by the utilization of other amino acids as nitrogen source. Growth rate of this mutant was not affected on minimal medium when glutamine, histidine, serine or lysine were used as a nitrogen sources. However, when arginine was used, the growth of the mutant show a very large *lag* (Fig. 2) These results suggest that the mutant strain require more time for arginine utilization, that may be due by induction of an alternative catabolic route.

Fig. 1

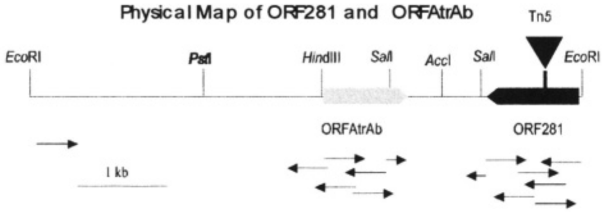
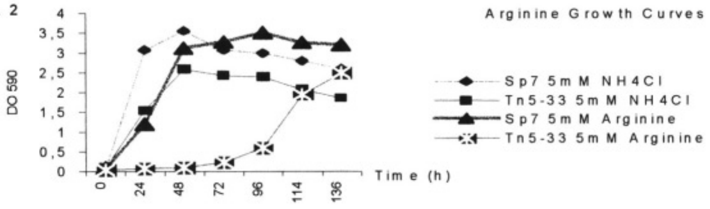


Fig. 2



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AMTB IS NECESSARY FOR NH_4^+ INDUCED NITROGENASE SWITCH-OFF AND ADP-RIBOSYLATION IN *Rhodobacter capsulatus*

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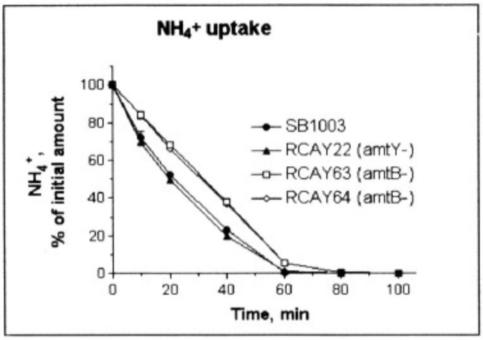
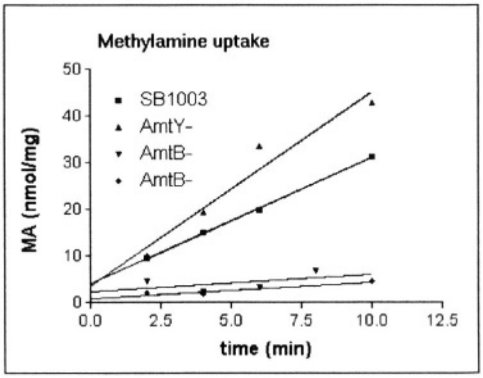
An analysis of the complete genome sequence of *Rhodobacter capsulatus* shows that it possesses two genes potentially coding for ammonia transporters, *amtB* and *amtY.* *amtY,* found a short distance from *ntrBC* and *nifR3,* is monocistronic whilst *amtB* is part of a *glnKamtB* operon as in many other bacteria. The conserved *glnKamtB* arrangement suggests that they may work together in regulating cellular processes in response to NH_4^+. The work reported here suggests that AmtB is presumably the high affinity ammonia transporter previously described. AmtB and AmtY are only distantly related and are predicted to have different membrane topologies.

To better understand the role of the two encoded proteins in the physiology of this bacterium and their possible significance in nitrogen fixation, we have created single “knock-out” mutants using insertional mutagenesis and subsequent recombination into the bacterial genome. Strains mutated in either *amtB* or *amtY* do not show a growth defect under any condition tested, including growth with N_2 as the sole nitrogen source. These results are in agreement with previous studies which have failed to find a growth phenotype for *amt* mutants at neutral pHs (D Meletzus et al. 1998, N Michel-Reydellet et al. 1998, E Soupene et al. 1998). In addition, strains mutated in either *amtB* or *amtY* do not excrete NH_4^+ and are still capable of transporting ammonia at nearly wild-type rates. (Further studies are in progress to ascertain the phenotype of a strain mutated in both *amtY* and *amtB.*) At the NH_4^+ concentration used here (1mM), *amtB* mutants showed a lower initial rate of ammonium uptake again suggesting that it corresponds to the high affinity system.





However, the *amtB* mutant is no longer capable of transporting methylamine while this activity is unaffected in the *amtY* strain. This finding is similar to what has been found for some other bacteria containing more than one Amt (M L Montesinos et al. 1998).

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Previously, we have shown that the short term responses to NH_4^+ addition are different depending upon the degree of cellular nitrogen limitation. Since the degree of nitrogen limitation affected the amount of the high affinity ammonia transport system which was present, we hypothesized that this system was responsible for initiating the response (A F Yakunin and P C Hallenbeck 1998). Here we have shown that in the short-term, the *amtB* strain, but not the *amtY* strain, in addition to being ineffective in methylamine uptake, is also completely defective in carrying out ADP-ribosylation of Fe-protein or otherwise modulating *in vivo* nitrogenase activity in response to NH_4^+ addition. This appears to be the first observation of a clear phenotype for an *amt* mutant at neutral pH. However, ADP-ribosylation in response to darkness is unaltered in the *amtB* strain. As well, the ADP-ribosylation of Fe-protein seen in response to different steady state cellular nitrogen levels (A F Yakunin et al. 1999) is unaffected. These results demonstrate that the machinery necessary for the ADP-ribosylation of Fe-protein is intact in this mutant which thus seems to be specifically affected in the signaling the presence of extracellular NH_4^+.

Interestingly, the *amtY* mutant shows higher levels of ADP-ribosylation than the wild type (SB 1003) strain in response to the addition of the same amount (200 µM) of NH_4^+. In addition, this mutant manifests an altered response to cellular nitrogen status as shown by studies where the initial NH_4^+ concentration was varied. Under these conditions, ADP-ribosylation occurs at lower initial NH_4^+ concentrations than in the wild type and a higher NH_4^+ concentration is required to repress nitrogenase synthesis. This suggests that the lack of AmtY shunts additional NH_4^+ through AmtB thus rendering this strain more sensitive to this effector.

The results obtained to date can be explained by a model in which AmtB and AmtY have different cellular functions. AmtY could function as a low-affinity, high capacity transporter (or permease (E Soupene et al. (1998)) and AmtB a high-affinity, low capacity transporter or permease. As previously noted, the loss of either one does not overly perturb overall NH_4^+ uptake. Since AmtB mutants obviously obtain enough NH_4^+ for growth, and take up NH_4^+ at rates that are nearly normal, the internal NH_4^+ pool must be nearly the same in this strain as in the wild-type. However, as shown here, AmtY can not substitute for AmtB in regulating nitrogenase in response to external NH_4^+. This suggests that the primary function of AmtB is to act as an ammonia sensor for the processes that regulate nitrogenase activity, perhaps by interacting in some manner with GlnK.

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Acknowledgements

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REGULATION OF *AZOTOBACTER VINELANDII* NIFA ACTIVITY BY NIFL: ROLE OF PII-LIKE PROTEINS IN NITROGEN SENSING

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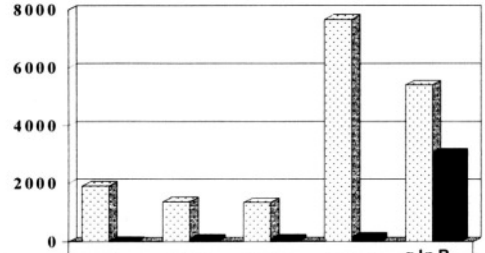
1. Introduction

The phenotypes of *nifL* mutants in *Azotobacter vinelandii* suggest that, in response to elevations in N status, NifL inhibits *nif*-specific transcription by NifA, and that this response may involve the product of *glnD* (Contreras et al,1991). Since *glnD* mutants are Nif and mutations in *nifL* suppress this phenotype, the uridylylation state of a PII-like regulatory protein could influence the inhibitory function of NifL. Unlike many other representatives of the proteobacteria, *A. vinelandii* carries only one copy of a gene encoding a GlnB/GlnK paralogue, referred to as *glnK,* which, unlike *Escherichia coli glnK,* is expressed constitutively (Meletzus et al,1998). Determination of the role of *A. vinelandii glnK* gene product in nitrogen signalling has been thwarted by the essential nature of this gene and it has not been possible to isolate insertion mutations in *glnK* (Meletzus et al,1998). In order to further investigate the mechanism of nitrogen signalling by *A. vinelandii* NifL, we have exploited characterised nitrogen regulatory mutants of *E. coli* to examine NifL and NifA function *in vivo* and have performed complementary experiments with purified proteins *in vitro.*

2. Influence of *E.coli glnB* and *glnK* mutations on the *A. vinelandii* NifA-NifL regulatory system

We have shown previously that the *Azotobacter vinelandii* NifL-NifA regulatory system is responsive to the redox and fixed N status in an *E. coli* wild type background (Söderbäck et al, 1998). Recently, enteric *glnK* was shown to be required for relief of inhibition by *K. pneumoniae* NifL *in vivo* under N-limiting conditions (He et al,1998; Jack et al,1999). However, neither *glnK* nor *glnB* mutations markedly increased the inhibition of *A. vinelandii* NifA activity by NifL under anaerobic N limitation, using a *nifH-lacZ* translational fusion as a reporter (Fig1). To check that the slight decrease in *nifH-lac* expression was not a general physiological effect, we included a *ntrC* mutant and a strain carrying mutations in both *ntrC* and *glnB.* In contrast, the induction was greater than in the wild type, indicating that the products of neither *glnK* nor *glnB* are required for derepression. Further, their absence alleviated repression by excess N (Fig 1).Thus, unlike the *K. pneumoniae* NifL-NifA system where GlnK apparently has a positive role in relieving inhibition by NifL under N-limiting conditions, the inhibitory function of *A vinelandii* NifL is activated under N-excess conditions



	wt	glnB	glnK	ntrC	glnB, ntrC
▨ -N	1921	1377	1357	7672	5415
■ +N	23	107	103	173	3072

Fig1 Transcriptional activation of a *nifH-lacZ* fusion in response to *A. vinelandii nifL* and *nifA* (β-galactosidase activity on the y axis) in various strains of *E. coli* (x axis). A corresponding data table is also shown beneath. Light bars indicate anaerobic N-limiting conditions, black bars indicate anaerobic N-excess conditions.

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through interaction with PII-like regulatory proteins in *E. coli.*

3. PII specifically increases NIFL inhibition in the presence of 2-ketoglutarate and ATP

We have previously shown that the inhibitory activity of NIFL on NIFA is stimulated by presence of adenosine nucleotides (Eydmann et al 1995) and that ADP increases the stability of the NIFL-NIFA protein complex (Money et al 1999). To determine the effects of PII and GlnK we used a truncated form of NIFL, NIFL (147-519), which responds to N but not O_2 *in vivo* and remains responsive to ADP *in vitro* (Söderbäck et al,1998), thus permitting assays to be done aerobically. The work of Ninfa's group has shown that both ATP and 2-ketoglutarate bind to *E. coli* PII and GlnK (Atkinson and Ninfa,1999; Kamberov et al,1995). However in control experiments in the absence of PII paralogues, we observed that 2-ketoglutarate is an effector of NifL activity, releasing inhibition in the presence of ATP or ADP (data not shown). This release was specific for 2-ketoglutarate, other keto acids and amino acids were ineffective. The effect of adding *E. coli* PII was to counteract the relief from inhibition by 2-ketoglutarate, whereas addition of either PII-UMP or *E. coli* GlnK was ineffective (Fig. 2). Purified *A. vinelandii* GlnK behaved similarly to *E. coli* PII, also activating the inhibitory function of NifL (data not shown).

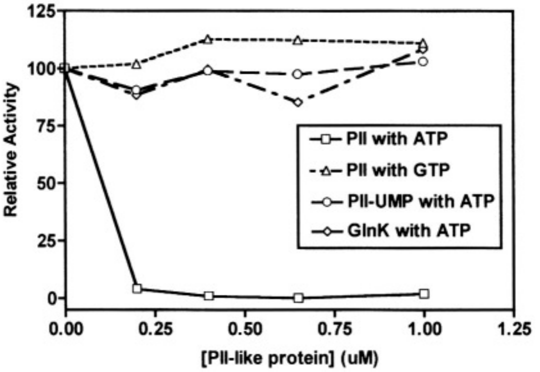

Fig 2. Influence of *E.coli* PII-like proteins on inhibition of NIFA activity by NIFL (147-519) *in vitro*. Assays contained 0.1mM 2-ketoglurarate, either 3.5 mM ATP, 0.5mM GTP (with ATP) or 4 mM GTP (with GTP), 50 nM NIFA, 100 nM NIFL and the indicated concentration of PII paralogue (squares, PII with ATP; triangles, PII with GTP; circles, PII-UMP with ATP; diamonds, GlnK with ATP). NIFA activity was determined by open promoter complex formation as described (Eydmann et al,1995).

The need for 2-ketoglutarate alone to lift ADP inhibition by *A. vinelandii* NifL and the increased inhibition seen in the presence of PII are consistent with our *in vivo* data using the *E. coli* mutant backgrounds reported above. In our current model for N sensing in *A. vinelandii* the interaction between NifL and NifA is decreased by elevated 2-ketoglutarate at low N status, and increased by the deuridylylation of GlnK at high N status.

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STRUCTURAL BASIS FOR SIGNAL TRANSDUCTION WITHIN THE FixJ TRANSCRIPTIONAL ACTIVATOR

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The '2-component' FixJ transcriptional activator is essential for nitrogen fixation in *Sinorhizobium meliloti* since it is required for expression of the *nifA* and *fixK* genes, which in turn activate fix and *fix* genes (David et al, 1988; Batut et al, 1989). FixJ is a modular protein containing a phosphorylatable receiver domain FixJN and a transcriptional activator domain FixJC whose activity is inhibited by FixJN within the native protein (Kahn & Ditta, 1991; Da Re et al., 1994). Two classes of models have been proposed to describe how phosphorylation of two-component regulators could activate a response. In one class of models the receiver domain acts as an inhibitory module, and phosphorylation triggers a conformational change in the receiver domain which abolishes this inhibition. This model was previously shown to apply to FixJ (Kahn & Ditta, 1991; Da Re et al., 1994), DctdD (Lee et al., 1994), Spo0A (Ireton et al., 1993) and CheB (Simms et al., 1985). It is supported by the crystallographic structures of CheB (Djordjevic et al., 1998) and NarL (Baikalov et al., 1996), in which receiver domains are found to sterically obstruct active sites in the output domains. In the second model class, phosphorylation of the receiver domain activates the response regulator by triggering its oligomerization as ,e.g., for NtrC (Porter et al., 1993) and OmpR (Huang et al., 1997). These two models are not mutually exclusive. Phosphorylation of FixJ triggers both the opening up of the molecule and its dimerization (Da Re et al., 1999).

1. Interaction between FixJN and FixJC within the FixJ Molecule

Phosphorylation triggers FixJ dimerization through the autonomous dimerization of the receiver domain (Da Re et al., 1999). Accordingly, the phosphorylation of both FixJ and FixJN are second order reactions with respect to protein concentration. However the second order phosphorylation constant is 44-fold higher for FixJN than for FixJ, which implies that the FixJC domain inhibits the chemical phosphorylation of the receiver domain within the FixJ molecule. Conversely FixJN had been shown previously to inhibit FixJC activity ca. 40-fold (Da Re et al., 1994). We interpret this mutual inhibition between FixJN and FixJC as a direct consequence of an interaction between the two domains, with a relatively weak energy $\Delta G = RT \ln 40 = 2.2$ kcal/mole. Such a weak interaction implies that native FixJ would exist in an equilibrium between a major closed inactive form and a minor open active form. In this model only the open form (ca. 2.5% of the total protein) would be active, which accounts for the residual activity which is indeed observed at high concentration of non phosphorylated FixJ (Reyrat et al., 1993; Da Re et al., 1994). The weak interaction energy between the two domains appears functionally relevant because it allows for the structure to open up at a moderate cost upon phosphorylation.

2. Alanine Scanning Mutagenesis of the FixJ Receiver Domain

We have used alanine scanning mutagenesis of the FixJ receiver domain in order to map its functional interfaces. Solvent exposed residues were predicted on the basis of the homologous structure of CheY (Stock et al., 1989), which led to the choice of 11 sets of residues to be mutated to alanine. Mutated proteins were purified as GST fusion proteins, the GST moiety was cleaved away with thrombin and mutated proteins purified. These proteins were tested for their ability to be

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phosphorylated, to dimerize, to bind DNA and to activate transcription. This analysis led to the identification of helix α4 and strand β5 as crucial elements for signal transduction in FixJ.

3. The FixJ Dimerization Interface involves Helix α4

The V91A and K95A mutations did not affect phosphorylation directly as shown by IEF and phosphorylation with radio-labeled acetyl-phosphate. However the phosphorylated proteins remained monomeric, so that the phosphorylation reaction is a 1st order reaction, contrary to wild-type FixJ which follows 2nd order. Therefore Val 91 and Lys 95 are essential for the phosphorylation-induced dimerization of FixJ. These results map the FixJ~P dimerization interface to helix α4 of the receiver domain, in agreement with structural data obtained recently by the group of Jean-Pierre Samama (Institut de Pharmacologie et de Biologie Structurale, Toulouse).

4. Phe-101 is Crucial to Signal Transduction between Phosphorylation Site and Output Domain

The F101A mutation strongly stimulates both DNA binding of unphosphorylated FixJ and transcription of the *nifA* promoter. This up phenotype suggests that the mutated FixJ protein adopts an open conformation liberating the activity of the C-terminal domain. Consistent with this hypothesis, F101A FixJ behaves abnormally on gel permeation chromatography, indicating an extended conformation. In addition the F101A FixJ protein cannot be phosphorylated with acetyl-phosphate, suggesting a propagating conformational change between F101 and the phosphorylation site. This dual phenotype of the F101A mutation indicates that Phe 101 interacts both with the phosphorylation site and the C-terminal domain. Thus Phe 101, which is generally conserved as an aromatic residue in response regulators, lies at the heart of signal transduction between the phosphorylation site and the output domain.

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EFFECT OF REDOX STATUS OF DINITROGENASE REDUCTASE ON THE REGULATION OF NITROGENASE ACTIVITY BY REVERSIBLE ADP-RIBOSYLATION

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1. Introduction

Nitrogenase activity in the phototrophic bacterium *Rhodospirillum rubrum* and related organisms is regulated by the reversible ADP-ribosylation of the dinitrogenase reductase protein at Arg 101. ADPR-ribosylation is catalyzed by the Dinitrogenase Reductase ADP-ribosyl Transferase (DRAT), and the removal of ADPR is catalyzed by Dinitrogenase Reductase Activating Glycohydrolase (DRAG). The ADPR-ribosylated form of the protein is unable to bind to dinitrogenase (the MoFe protein) and thus is unable to transfer electrons.

The current model for the regulation is shown in Fig 1. In vivo, in response to darkness or addition of fixed nitrogen to the medium, the ADP-bound form of the enzyme is subject to ADPR-ribosylation by DRAT. When the culture is re-illuminated, DRAG carries out the activation of ATP-bound dinitrogenase reductase. In this paper, data is presented to support an additional feature of the model: that the redox status of dinitrogenase reductase affects its ability to be acted upon by DRAT or DRAG. In this new model, DRAT functions only on the oxidized form of the protein, while DRAG acts on the reduced form of dinitrogenase reductase. Evidence for a role of GlnB (PII) is also presented.

2. Procedures and Results

Purified dinitrogenase reductase was prepared in dithionite-reduced and in thionine-oxidized forms in an anaerobic glove box. Redox dye-mediated titrations of dinitrogenase were performed using benzyl viologen, and the redox status of dinitrogenase reductase was determined by the absorption at 430 nm. When the ability of ADP-ribosylated dinitrogenase reductase to be activated by DRAG in reaction mixtures set at various redox potentials was compared to the redox status of the Fe_4S_4 cluster of the protein, the data points fell on a very similar curve (Fig. 2). From this result, we conclude that DRAG can only remove ADP-ribose from the reduced form of dinitrogenase reductase. It is important to note that DRAG activity with low molecular weight substrates such as ADPR-dansyl arginine methyl ester is unaffected by redox status of the reaction mixture across the range shown in Fig 2 (data not shown), and thus we conclude that the redox effect is on the substrate (dinitrogenase reductase) rather than on DRAG itself.

Effect of Redox on DRAT. In a separate experiment, it was found that DRAT is effective in the NAD-dependent ADP-ribosylation of oxidized dinitrogenase reductase, but ineffective in the modification of the reduced protein (data not shown). DRAT and

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Fig. 1

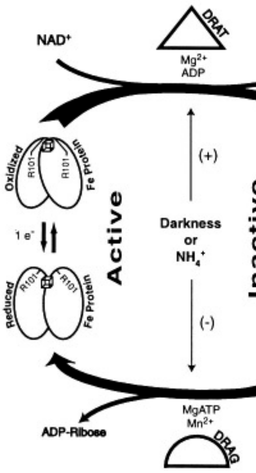
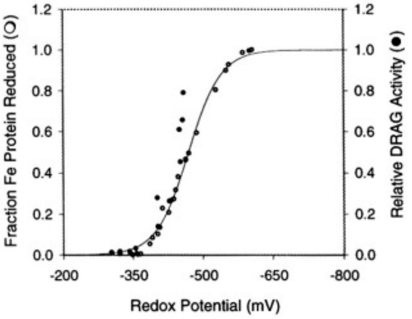


Fig. 2

DRAG Activity Redox Dependence



DRAG activities were determined by decoupled DRAG assays in 1 mM benzyl viologen (●), with redox potentials measured by direct potentiometry. The fraction of Fe protein reduced (○) at poised potentials was determined from the change in the characteristic Fe protein absorbance at 420 nm. Redox potentials were determined from the intensity of the absorption at 608 nm due to reduced MV.

DRAG appear to have opposite specificities for redox status of their target forms of dinitrogenase reductase. When a *nifD* strain of *R*, *rubrum* was tested for dark or ammonium-dependent in vivo ADP-ribosylation of dinitrogenase reductase, it was found that ADP-ribosylation and loss of activity occurred very slowly. This is interpreted as evidence that DRAT is less able to modify dinitrogenase reductase in a *nifD* strain (lacks dinitrogenase) because the electron acceptor for dinitrogenase reductase is absent and the protein remains reduced (data not shown).

Role of GlnB (PII) in regulation of DRAT and DRAG activities. GlnB mutants of *R*, *rubrum* do not express *nif* gene products. However, if a plasmid encoding *Klebsiella pneumoniae* NifA is introduced, *nif* gene expression in the *glnB* background is observed. These strains are impaired in ammonia-stimulated ADP-ribosylation of dinitrogenase reductase in vivo, but the dark-dependent ADP-ribosylation is not affected. A *glnK* paralog of *glnB* has been identified in *R. rubrum,* but mutations in this gene have no effect on *nif* expression or the function of DRAT and DRAG in *R. rubrum.*

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HETEROTRIMERIZATION OF P_{II}-LIKE SIGNALLING PROTEINS: LESSONS FROM A COMPARATIVE ANALYSIS BETWEEN A CYANOBACTERIAL P_{II} HOMOLOGUE AND ITS PROTEOBACTERIAL COUNTERPARTS

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1. Introduction

The *glnB* gene family, which encodes the P_{II} signalling protein, is present in representatives of all three domains of life. Thus, the P_{II} protein is considered as one of the most highly conserved signaling proteins known so far. The P_{II} protein in *E. coli* (*Ec*P_{II}), which is studied since three decades, represents a paradigm for signal transduction cascades. A homologue of this P_{II} protein has been identified in the cyanobacterium *Synechococcus* PCC 7942 (*Sc*P_{II}) (Tsinoremas et al. 1991). The 12,4 kDa P_{II} subunit shares 65 % amino acid identity to the *E. coli* counterpart and like this, its quaternary structure is trimeric (Forchhammer, Tandeau de Marsac, 1994). In contrast to *Ec*P_{II}, the cyanobacterial homologue is not modified by uridylylation at Tyr 51, instead, its seryl residue 49 is phosphorylated (Forchhammer, Tandeau de Marsac, 1995). Like the uridylylation state of *Ec*P_{II}, the phosphorylation state *Sc*P_{II} can be revealed by non-denaturing PAGE followed by immunological detection (Fig. 1). *Sc*P_{II} phosphorylation responds to the cellular state of nitrogen and carbon assimilation. In vitro studies showed a fundamental functional conservation between the P_{II} proteins from *E. coli* and *Symchococcus*: Both proteins bind the metabolites ATP and 2-oxoglutarate in a synergistic manner with the binding constants being in the same order of magnitude (Kamberov et al., 1995; Forchhammer, Hedler, 1997). Whereas *Ec*P_{II} is reversibly uridylylated by a bifunctional enzyme, the reactivity of which is governed by the cellular glutamine level (Jiang et al., 1998), phosphorylation of *Sc*P_{II} is controlled by the 2-oxoglutate level. The P_{II} kinase, which is still unknown at the genetic level, utilizes ATP as phosphoryl donor and requires 2-oxoglutarate-bound *Sc*P_{II} as substrate (Forchhammer, Tandeau de Marsac, 1995). Conversely, dephosphorylation of phospho-*Sc*P_{II} is carried out by a protein-serine- phosphatase, which only recognizes phospho-*Sc*P_{II} that is devoid of 2-oxoglutarate (Irmler et al. 1997).

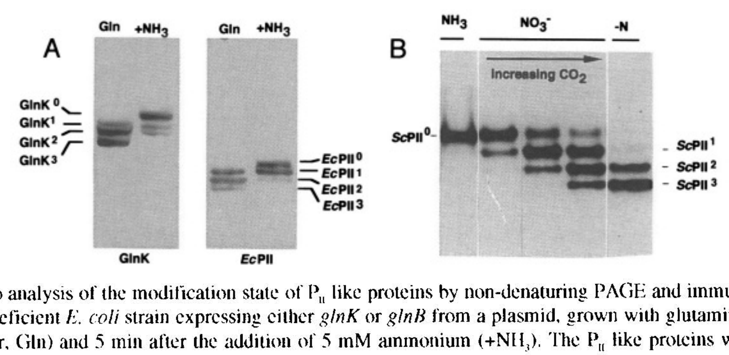

Fig. 1: In vivo analysis of the modification state of P_{II} like proteins by non-denaturing PAGE and immunoblotting. A: A *glnBglnK* deficient *E. coli* strain expressing either *glnK* or *glnB* from a plasmid, grown with glutamine as N-source (nitrogen-poor, Gln) and 5 min after the addition of 5 mM ammonium (+NH_3). The P_{II} like proteins were visualized with a antiserum raised against GlnK. B: *Synechococcus* wild-type cells grown in the presence of ammonium, nitrate (with different CO_2 supplies) or starved for nitrogen. The numbers of modified subunits per trimeric P_{II} proteins is indicated.

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2. Compatibility of the cyanobacterial and the *E. coli* P_{II} signal transduction systems

Expression of the *Symchococcus glnB* gene in *E. coli* leads to a profound perturbation of the *Ec*P_{II} signalling system (Forchhammer, Hedler, 1997; Forchhammer et al., 1999). *E. coli* cells synthesizing *Sc*P_{II} are unable to repress glutamine synthetase production under conditions of nitrogen excess and are unable to deadenylylate glutamine synthetase following a transfer from nitrogen excess to nitrogen poor medium. To elucidate the molecular basis of this effect, we investigated possible interactions between *Sc*P_{II} and the *E. coli* homologues. Co-immunoprecipitation experiments showed that the *Sc*P_{II} subunits form complexes with *Ec*P_{II} and with the *E. coli* P_{II} paralogue GlnK. Analysis of these complexes by non-denaturing PAGE and immunoblotting (employing antisera which are specific either to *Sc*P_{II} or to *Ec*P_{II} and GlnK) revealed the formation of heterotrimers between these phylogenetically distinct P_{II} homologues (Forchhammer et al., 1999). A further refinement of this analytical method allowed us to study the modification of the different heterotrimers by uridylylation following ammonium treatment. Although uridylylation of the heterotrimers was still possible, it was much slower than that of homotrimeric *Ec*P_{II} or GlnK. We could also provide evidences that homotrimeric *Sc*P_{II} could be uridylylated in *E. coli* cells, although at a very low level.

3. Conclusions for P_{II} signal transduction systems with multipleP_{II} paralogues

From the fact that phylogenetically distinct P_{II} proteins like these of *E. coli* and *Synechococcus* are able to form heterotrimers, it can be concluded that the subunit interaction is highly conserved in these proteins. It was thus tentative to speculate that the P_{II} paralogues in *E. coli* (*Ec*P_{II} and GlnK) form heterotrimers with each other. By coexpressing the respective genes in a *glnD* deficient background (in which the proteins are not modified by uridylylation) we could reveal electrophoretic forms of P_{II} cross-reactive material which displayed all characteristics of *Ec*P_{II}/GlnK heterotrimers. This strongly supports our suggestion that the P_{II} paralogues form heterotrimers with each other. The in vivo analysis of signal transduction by *Ec*P_{II} in heterotrimers with *Sc*P_{II} showed that the function of *Ec*P_{II} is severely impaired in heterotrimers Ninfa and co-workers suggested that the T-loop of a single P_{II} subunit interacts with the P_{II}-receptors NtrB and GlnE (Jiang et al., 1997). According to this, the conformation of the T-loop would be affected by the subunit partner of P_{II} in such a manner, that functional interactions with the receptors are altered. Since the formation of heterotrimers affects the function of the P_{II} signal transduction system, differential expression of P_{II} paralogues would superimpose a novel regulatory level on the P_{II} signal transduction systems by heterotrimerization.

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ROLE OF PII PROTEIN AS A SIGNAL NITROGEN LEVEL IN *HERBASPIRILLUM SEROPEDICAE*

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1. Introduction

The PII protein is essential for nitrogenase expression in *Herbaspirillum seropedicae,* probably by sensing the intracellular nitrogen levels and relaying a signal to NifA, the *nif* specific transcription activator. A *glnB* gene insertional mutant of *H. seropedicae* had a Nif^- phenotype and, although the *nifA* gene was expressed, the *nifB* gene was not expressed in this mutant, suggesting that PII is necessary for NifA activity in *H. seropedicae. H. seropedicae* PII is not involved in the control of GS activity by adenylylation or in its biosynthesis.

2. PII uridylylation/deuridylylation assays

Uridylylation assays of *H. seropedicae* PII under conditions of low and high nitrogen availability with purified *E. coli* GlnD protein or a cell-free extract of *H. seropedicae* indicated that the pattern of uridylylation was similar, but not identical, to that of other organisms, α-ketoglutarate and ATP were not an essential requirement for the uridylylation of PII of *H. seropedicae.* In the presence of either 0.1 mM ATP or 10 mM α-ketoglutarate the native PII was uridylylated at a rate of 25% or 65%, respectively, of the maximum rate obtained in the presence of both α-ketoglutarate and ATP. This result suggests that these effectors bind PII co-operatively as seen with the *E. coli* and *Synechococcus* PII proteins (Kamberov et al, 1995; Forchhammer and Hedler, 1997). Therefore, the uridylylation requirement of the *H. seropedicae* PII was different from that of the PII of *E. coli*, where the uridylylation was reported to be absolutely dependent on the presence of both ATP and α-ketoglutarate (Kamberov et al, 1994).

The PII uridylylation was inhibited by glutamine. Glutamine inhibited the uridylyltransferase activity of the *E. coli* GlnD at lower concentration than that of *H. seropedicae* GlnD (50% inhibition at 200 μM compared to 1 mM), suggesting that nitrogen excess is signalled at lower concentration of glutamine in *E. coli* than in *H. seropedicae.* This may reflect glutamine consumption by the extract.

No deuridylylation was observed upon addition of glutamine. The PII-[^{32}P]UMP was recovered from a uridylylation reaction by dialysis and used in deuridylylation assays in the presence of Mn^{2+}. PII-UMP was deuridylylated by an *H. seropedicae* cell-free extract in the presence of glutamine but further addition of ATP (0.1 mM) and α-ketoglutarate (0.1 mM) inhibited deuridylylation.

3. *H. seropedicae* PII structure

The PII protein of *H. seropedicae* was crystallised. *H. seropedicae* PII protein is a trimer as is the *E. coli* PII and its monomer contains a double βαβ motif, the T loop with Tyr51, the B loop and the C loop. On the trimer the β-sheets are around a central cavity and the α-helix on the

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outer surface of the protein. In the trimer a lateral cleft is formed between the monomers. This cleft is surrounded by the C, T and B loops. Comparing proteins of both organisms, the major structural difference was found in the C-terminal region: *H. seropedicae* PII contains a **3_{10}-helix,** not present in *E. coli* PII. However, a similar structural motif occurs in the *E. coli* GlnK protein. Indeed, the *H. seropedicae* PII appears to be functionally similar to the enteric GlnK, relieving NifA inhibition as GlnK does with NifL *of K. pneumoniae* (He et al, 1998; Jack et al, 1999). These results suggest that the C-terminal region of *H. seropedicae* PII is may be involved in signalling the nitrogen level to NifA.

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MODIFICATION OF THE PII PROTEIN IN RESPONSE TO NITROGEN AVAILABILITY IN FILAMENTOUS HETEROCYSTOUS CYANOBACTERIA *ANABAENA SP.* PCC 7120.

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The *glnB* gene product PII, present in bacteria, archae and plants, is able to sense the availability of nitrogen and carbon source, and accordingly, regulates nitrogen acquisition and assimilation at multiple levels. When fixed nitrogen is limiting, many strains of filamentous cyanobacteria such as *Anabaena sp.* PCC 7120, can also differentiate specialized cells called heterocysts in which nitrogen fixation occurs. This differentiation process is regulated by combined-nitrogen availability.

The *glnB* gene of *Anabaena sp.* PCC 7120 was isolated from a genomic library and cloned into the His-tag vector pET15b. The PII His-tag protein was over-produced in the *E. coli* DE3 and purified to apparent purity with a metal-chelating affinity column (Novagen). The purified recombinant protein PII of 12,6 kD, when loaded onto a gel filtration column, could be eluted as a protein with an apparent molecular weight of 44 kD. These results suggest that PII forms a homotrimer.

The native PII and recombinant PII proteins were modified in cells cultured in the presence of nitrate, but not in cells cultured in the presence of ammonium. Consistent with previous results, four forms of PII could be resolved (by non-denaturing polyacrylamide gel electrophoresis) from filaments cultured in the presence of nitrate: one non-modified (PII0), and three modified forms (PII1, PII2, PII3) corresponding to cases where one, two, or three subunits were modified. The PII modification during heterocysts development showed that half-hour after the transfer of filaments to a nitrogen free medium (BG110), PII was totally unmodified. Six hours after the transfer, modified forms of PII appear again, and this situation lasted until 24 hours at time when heterocysts started to be visible under light microscope. After 36 hours, when nitrogen fixation was fully operational, only weakly modified form PII could be seen, in a situation similar to that of filaments continously cultured in BG110. These experiments demonstrated a dynamic pattern of PII modification during heterocysts differentiation

The modification of PII protein from *Anabaena sp.* PCC 7120 occurs in the form of phosphorylation on a Ser residue as well as in the PII protein from *Synechocystis sp.* PCC 6803. Although, the PII protein from *Synechocystis sp.* PCC 6803 migrated faster than that from *Anabaena sp* PCC 7120 in a non-denaturing polyacrylamide gel, and when cells were transferred to a nitrate-containing BG11 medium, PII from *Synechocystis sp.* PCC 6803 became strongly modified while this did not occur even 4 hours after the transfer for the PII protein from *Anabaena sp.* PCC 7120. In addition, the PII protein seems to be essential in *Anabaena sp.* PCC 7120 since inactivation of *glnB* gene was unsuccessful under all conditions tested so far.

EFFECT OF *RHIZOBIUM* RESPIRATORY MUTANTS ON THE REGULATION OF CARBON AND NITROGEN ASSIMILATION IN *PHASEOLUS VULGARIS* NODULES.

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Symbiotic reduced nitrogen in legumes root nodules, is assimilated via the incorporation of NH_4^+ into the amide position of glutamine in a reaction catalyzed by glutamine synthetase (GS). In temperate legumes, the assimilated nitrogen is transported from the nodules to the shoot in the form of amides (glutamine and asparagine), whereas nodules of tropical legumes export ureides (allantoin and allantoic acid). Ureides comprise 70 to 95% of total organic nitrogen in the xylem sap when these plants are growing symbiotically with N_2 fixing microorganisms. It has been proposed that the abundance of ureides in the plant shoot axis may provide a quantitative assay of N_2 fixation (Herridge 1982; Mc Clure and Israel 1979). In contrast, the sensitivity of N_2 fixation to drought stress has been shown to be associated with the ureide accumulation in the shoot, which has led to the hypothesis that N_2 fixation during drought is decreased by a feedback mechanism involving N metabolism and the pool of reduced N in the plant (Silsburry et al., 1986; Parson et al., 1993; Hartwig et al., 1994; Serraj et al., 1999). Moreover ureide applied to N_2 fixing plants resulted in an almost complete inhibition of nitrogenase which is accompanied by an increase in ureide concentration in the shoot and in a continual decrease in nodule permeability to O_2 (Serraj et al., 1999). Also it has been proposed that N_2 fixation inhibition by a range of environmental perturbation could be mediated by both the nodule permeability to O_2 and the potential to metabolize sucrose (Gordon et al., 1997).

This work further explore the relationship between nodule O_2 consumption *Rhizobium* respiration and the ureide synthesis and accumulation in the shoot, without imposing any perturbation which disrupt neither plant integrity nor nodule environmental conditions. We analyzed the activities of xanthine dehydrogenase (XDH), uricase, allantoinase, glucose-6-phosphate dehydrogenase (G6PDH), and aspartate amino transferase (AAT) in bean nodules, inoculated with the wild type *Rhizobium etli* strain CE3 and its derivative CFN037 with increased respiratory capacity. Plants inoculated with the *Rhizobium* mutant CFN037 had lower ureide content in the xylem sap than the plants inoculated with the *Rhizobium* strain CE3. Supporting this, lower XDH, allantoinase, uricase and G6PDH were detected in nodules induced by the *Rhizobium* mutant CFN037. Moreover, these nodules showed the high glutamate synthase (GOGAT) and phospho*enol*pyruvate carboxylase (PEPc), activities and mRNA levels. The data presented here show that increased nodule O_2 consumption by increasing *Rhizobium* respiration reduced the synthesis and the transport of ureide, supporting the idea that N_2 fixation in legumes might be regulated by a feedback mechanism involving N compounds. These results also suggest that bacterial respiration may go beyond the obvious effects on ATP and NAD(P)H generation to some effects on the synthesis and transport of nitrogen solutes.

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IMMUNOGOLD LOCALISATION OF GLUTAMINE SYNTHETASE IN *MEDICAGO TRUNCATULA* ROOT NODULES

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The ammonium released by *Rhizobium* as a result of symbiotic N_2 fixation is assimilated mainly by the plant glutamine synthetase (GS). In higher plants, GS occurs as a number of isoenzymes located in the cytosol and plastids. However, few studies have been devoted to the cellular and subcellular localisation of GS within root nodules. Using immunogold electron microscopy, we have performed a detailed analysis of GS polypeptide distribution in different tissues of *Medicago truncatula* root nodules. In the central nodule tissues, GS was found in both the cytosol and amyloplasts of infected and uninfected cells. In peripheral nodule tissues, a striking abundance of GS was detected in the nodule vascular bundle pericycle cells, especially in transfer cells. We also detected high levels of GS in the cytosol and amyloplasts of the nodule parenchyma cells. The location and abundance of GS in the cytosol of the infected cells is entirely consistent with its major role in nodules of assimilating ammonium produced at high rates by the rhizobia. However, the detection and high abundance of GS in the plastids and cytosol of other nodule tissues (vascular bundles, parenchyma and uninfected cells) strongly suggests that GS may be involved in other physiological processes.

Transfer cells occur in the nodule vasculature of many amide-exporting species and play a role in transporting amino acids away from the infected nodule tissue to the xylem and in supplying photosynthate from the phloem to the infected cells (Pate *et al,* 1969). The noticeable abundance of GS detected in these specialised cells suggests an involvement of GS in this process. The detection of GS in the parenchyma and in uninfected cells suggests it as a component of mechanisms contributing to the regulation of the oxygen-diffusion barrier, by producting nitrogen-containing osmotic solutes. Many other enzymes involved in nitrogen and carbon metabolism have recently been located in the nodule parenchyma including aspartate amino transferase (Yoshioka et al, 1999), asparagine synthetase (Shi et al, 1997), PEP carboxylase (Pathirana *et al.,* 1997), carbonic anhydrase (de la Pena *et al,* 1997) and malate dehydrogenase (Miller *et al,* 1998). Thus, in parenchyma cells, GS may act in conjunction with other enzymes in a common metabolic pathway to produce organic compounds involved in osmotic changes important for the regulation of the oxygen-diffusion barrier. This work has also shown the presence of plastidial GS isoenzymes in almost all tissues analysed. As plastidial GS isoenzymes represent a significant part of GS activity in nodules of *M. truncatula* as well as in other species, it appears that it might play an important role in nodule metabolism but the nature of that role remains to be elucidated.

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LONG-TERM EFFECTS OF CO_2 ENRICHMENT ON NITROGEN FIXATION AND NODULE METABOLISM IN *Pisum sativum* L. PLANTS

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1. Introduction

It has been suggested that CO_2 enrichment may have beneficial effects on C3 plants, particularly on those having large sinks (Poorter, 1993), as the N_2-fixing plants. Indeed, it has been reported that CO_2 enrichment increases nodule mass, but there is scant information on the effects of long-term elevated CO_2 on nodule metabolism and specific nitrogen fixation. Here, we report nitrogen fixation analysis of pea nodules using an open flow-through system, together with a comprehensive analysis of nodule metabolism.

2. Material and Methods

Pea plants *(Pisum sativum* L. cv. Frilene) nodulated with *R. leguminosarum* bv. *viciae* were grown for 4 weeks in environmental-controlled chambers at 360 or 1000 $\mu mol\ mol^{-1}$ CO_2, as described elsewhere (Cabrerizo et al., 1998). Then, nodule harvest and carbohydrate and enzyme analysis were performed as in González et al. (1998). Organic acids were determined by ion chromatography and nitrogen fixation as described by Witty and Minchin (1998).

3. Results and discussion

At elevated CO_2, pea nodules showed higher concentrations of plant fraction protein and leghaemoglobin than in ambient CO_2, whilst we failed to detect any significant difference in bacteroid protein. Also, starch, sucrose and malate levels were higher in these nodules, although citrate levels were lower. A significant enhancement of sucrose synthase, UDPG pyrophosphorylase, PEP carboxylase, glutamine synthetase and isocitrate dehydrogenase activities was monitored in these nodules. Also, nitrogen fixation was higher in a plant basis. However, specific nitrogen fixation at both CO_2 was almost identical, this result being largely due to a lower EAC. It is concluded that the main effect of long-term effects of CO_2 enrichment on nitrogen fixation is on nodule mass, its effects on specific nitrogen fixation being almost negligible.

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PROTEIN:PROTEIN INTERACTIONS BETWEEN THE ENHANCER BINDING PROTEIN, NIFA AND THE SENSOR NIFL

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The enhancer binding protein NIFA and the sensor protein NIFL from *Azotobacter vinelandii* comprise an atypical two component regulatory system which regulates the expression of genes involved in nitrogen fixation. The NIFA protein activates transcription from σ^N-dependent *nif* promoters in combination with σ^N RNA polymerase holoenzyme and its activity is repressed by the NIFL protein in response to increases in the levels of fixed nitrogen and extracellular oxygen. We have shown that *Azotobacter vinelandii* NIFL is a flavoprotein with FAD as the prosthetic group (Hill et al 1996). The oxidised form of NIFL is competent to inhibit NIFA activity but reduction of the flavin moiety in NIFL abolishes its ability to inhibit NIFA. Thus NIFL acts as a redox-sensitive molecular switch to regulate NIFA activity. The inhibitory activity of NIFL is also stimulated by adenosine nucleotides *in vitro,* suggesting that it may sense energy charge *in vivo* (Eydmann et al 1996). We have shown recently that adenosine nucleotides, particularly ADP, promote complex formation between purified NIFA and NIFL allowing isolation and characterisation of the NIFL:NIFA complex *in vitro* (Money et al 1999). Thus in this system signal transduction occurs via protein-protein interaction rather than by a phosphotransfer mechanism. Protease footprinting experiments using full length and isolated domains of the proteins have now been employed to identify amino acid sequences involved in interactions between NIFL and NIFA during complex formation in the presence of MgADP.

Purified NIFA and NIFL proteins were subjected to limited trypsin digestion in the presence MgADP and peptides from the digested proteins were identified by western blotting with anti-NIFA or NIFL sera and by N-terminal sequence analysis. We have shown previously that the presence of MgADP protects both NIFL and NIFA individually from trypsin digestion. The central domain of NIFA and the C-terminal domain of NIFL are protected (S. Austin, unpublished, Soderback et al 1998). We now observe additional changes in the pattern of tryptic peptides when NIFA and NIFL are mixed together to form a complex in the presence of MgADP. The Q-linker regions of both proteins and a region of the ammo-terminal domain of NIFA immediately adjacent to the Q-linker are protected in the NIFL:NIFA complex. The changes in trypsin sensitivity in the linker regions could be due to direct protein:protein contacts by these surfaces of NIFL and NIFA or to conformational changes resulting from contacts in other regions of the proteins. Using truncated fragments and domains of NIFA we show that the isolated central domain of NIFA alone is sufficient to protect the Q-linker of NIFL from trypsin cleavage. We also show that the changes in trypsin sensitivity in the amino-terminus and Q-linker of NIFA correlate with the ability of the protein to form a stable complex with NIFL detectable by co-chromatography.

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UREA UTILIZATION IN *RHODOBACTER CAPSULATUS* IS REGULATED BY NTRC

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The phototrophic non-sulphur purple bacterium *R. capsulatus* can use urea as sole source of nitrogen. Five mutants (Xan-9, Xan-10, Xan-19, Xan-20, and Xan-22) unable to grow with urea as the only N source were isolated by random transposon Tn*5* mutagenesis (Leimkühler et al., 1998). Three Tn*5* insertion sites were mapped within the urease gene cluster (*ure*), whereas the remaining two mutations were located within the *ntrB-C* (*nifR2-R1*) operon. DNA sequence analysis of the *R. capsulatus ure* gene region demonstrated the presence of the *ureDABC-orf136-ureEFG* genes, which are assumed to form one transcriptional unit due to the close linkage of these eight genes. The deduced gene products of the *ure* genes show high similarity to corresponding proteins from other bacteria, whereas *orf136* seems to be specific for *R. capsulatus*, since homologues were neither found in other *ure* gene regions nor elsewhere in the databases.

Interposon mutagenesis proved that *ureD* and *ureG* are essential for urease activity. In contrast, only a polar mutation within *orf136* resulted in an Ure$^-$ phenotype, whereas a non-polar *orf136* mutation did not affect urease activity indicating that *orf136* itself is not essential for urea utilization but genes downstream of and cotranscribed with *orf136* are necessary for this process. Upstream of *ureD* two *lrp*-like genes (*lrp1*, *lrp2*) were identified, which are not required for urease activity. An open reading frame (*orf433*) downstream of *ureG* might code for a periplasmic protein, which exhibits some similarity to the FmdD protein of *Methylophilus methylotrophus*, which is thought to comprise part of a high-affinity, binding-protein-dependent active-transport system for short-chain amides and urea (Mills et al., 1998).

Analysis of an *ureDA-lacZ* fusion showed that maximum expression of the *ure* genes occured under nitrogen-limiting conditions (serine or urea as sole nitrogen source), but *ure* gene expression was not substrate (urea)-inducible. Expression of the *ure* genes was down-regulated (by factor 3.5) under nitrogen-replete conditions. Although *ure* gene expression was strictly dependent on NtrC, significant transcription could be observed in the presence of ammonium in an ***ntrC***$^+$ background. This might be an indication that the expression of the *ure* genes in the presence of ammonium somehow requires NtrC. Expression of the *ure* genes does not depend on RpoN (NifR4). The transcriptional start site of the *ure* operon was mapped by primer extension within the intergenic region between *lrp2* and *ureD.* The presence of two DNA sequences resembling putative NtrC binding sites upstream of the transcriptional start site suggest that NtrC directly regulates *ure* gene expression in *R. capsulatus.* However, although urea is used as a nitrogen soure in an NtrC-dependent manner, nitrogenase activity was not repressed by urea.

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STRUCTURE AND REGULATION OF PYRUVATE DEHYDROGENASE GENES FROM *SINORHIZOBIUM MELILOTI*

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Sinorhizobium meliloti is an **α-proteobacterium** that lives either as a free-living cell in the soil or as an intracellular symbiont plant of alfalfa and closely related legume species. The bacteria establish symbiosis by eliciting the formation of nodules on plant roots, in which they differentiate into bacteroids capable of fixing atmospheric nitrogen into ammonia to the benefit of the host plant (Mylona *et al.* 1995). Rhizobia are strict aerobes that made up the energy required for nitrogen fixation by operation of a tricarboxylic acid (TCA) cycle coupled to oxidative phosphorylation. In order to cope with the low ambient oxygen concentration of the nodule (10-30 nM O_2), rhizobia synthesize a specialised oxidase complex with high affinity for oxygen (Preisig *et al.* 1993). Expression of the corresponding *fixNOQP* operon is itself under **O_2-control** *via* a master two-component regulatory system, *fixLJ,* which also controls nitrogenase synthesis in *S. meliloti* (David *et al.* 1988). Contrary to free-living bacteria and to bacteria at the early stages of plant colonisation, nitrogen-fixing *S. meliloti* cells heavily depend on dicarboxylic acids (succinate, malate and fumarate) provided by the plant partner, as carbon sources. Because succinate, fumarate and malate are intermediates of the TCA cycle, operation of the latter requires anaplerotic synthesis of acetyl coenzyme A. It was proposed that acetyl-CoA is mainly generated in bacteroids from the combined activities of malic enzyme and pyruvate dehydrogenase (Driscoll, Finan. 1993). So, pyruvate dehydrogenase was postulated to be a key enzyme of carbon metabolism in bacteroids.

Genes coding for components of the large pyruvate dehydrogenase multienzyme complex (PDHc) from *Sinorhizobium meliloti* have been isolated on the basis of their high expression in symbiotic bacteria. The Elp component, pyruvate dehydrogenase (PDH), is encoded by two genes, ***pdhAα*** (1,047 bp) and ***pdhAβ*** (1,383 bp), a situation encountered in the **α-proteobacteria** *Rickettsia prowazekii* and *Zymomonas mobilis*, as well as in some gram positive bacteria and in mitochondria, ***pdhAα*** and ***pdhAβ*** precede *pdhB* (1,344bp) that encodes the E2p component, dihydrolipoamide acetyltransferase, of the PDHc. No gene encoding the E3 component, lipoamide dehydrogenase, was found in the immediate vicinity of *pdhA* and *pdhB* genes. ***pdhAα***, ***pdhAβ*** and *pdhB* likely constitute an operon. We provide evidence that *pdhA* expression is induced in the symbiotic stage as compared to free-living conditions. We demonstrate that symbiotic expression of *pdhA* genes does not depend on the *fixLJ* regulatory cascade that regulates nitrogen fixation and respiration gene expression in symbiotic *S. meliloti* cells. Induction of *pdhA* expression could be obtained under free-living conditions upon the addition of pyruvate to the culture medium. Induction by pyruvate and symbiotic activation of *pdh* gene expression take place at the same promoter.

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INVOLVEMENT OF THE *recX* GENE PRODUCT IN THE SOS RESPONSE OF THE DIAZOTROPH *HERBASPIRILLUM SEROPEDICAE.*

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Herbaspirillum seropedicae is an endophytic nitrogen-fixing bacterium found in the leaves, roots and stems of several Poaceae such as rice and sugar-cane (Baldani *et al*., 1986; Döbereiner, 1992). The potential contribution in fixed nitrogen to associated plant growth has motivated genetic and physiological studies of this microrganism.

In *Escherichia coli,* the RecA protein plays a central role in homologous recombination and DNA repair (De Mot *et al.,* 1994). A *recX* gene was found downstream of the *recA* gene in several species and although the function of RecX is not yet known, it is required for viability of the bacteria when RecA is overexpressed in *Pseudomonas aeruginosa* and *Mycobacterium smegmatis* (Sano, 1993; Papavinasasundaram *et al.,* 1998).

The *recA* gene of *H. seropedicae* was isolated, by complementation of a *recA* mutation of *E. coli* HB101, and cloned, yielding the plasmid pBMR5 (Steffens *et al.,* 1993). The 4.5 kb *Eco*RI/*Hind*III fragment from pBMR5, which hybridised with the *recA* from *E. coli* K12, was partially sequenced. Analysis of the sequenced 2.5 kb region, by the method of codon preference, indicated the presence of two complete and contiguous open reading frames (ORFs), corresponding to 352 and 154 amino acids. The comparison of the derived aminoacid sequence of ORF1, in the Genbank, revealed homology with the RecA proteins from *Burkholderia cepacia* (identity 81%, similarity 90%), *Bordetella pertussis* (identity 76%, similarity 84%) and *Methylomonas fluorescens* (identity 78%, similarity 87%), among several others. The ORF2 shows homology with the RecX proteins from *P. fluorescens* (identity 40%, similarity 53%), *P. aeruginosa* (identity 37%, similarity 54%) and *E. coli* (identity 31%, similarity 45%), among others. The *recX* DNA sequence *of H. seropedicae* was deposited in Genbank under the access number AFO84045. A putative σ^{70} promoter was identified upstream of *recA* and a terminator sequence was identified downstream of *recX.* The intergenic region contained no promoter. A *lacZ*-Km cassette was introduced into the *recX* coding region, producing the plasmid pCWG1. Site-directed mutagenesis of the *H. seropedicae* SMR1 strain produced a mutant (CWG1) sensitive to methyl methanesulfonate and partially complemented with the plasmid pBMR26.2 **(*recA*$^-$ *recX*$^+$).** These results confirm the presence of the *recA* and the *recX* genes, organized in the same operon, and support the involvement of the RecX protein in the SOS response in *H. seropedicae.*

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MOLECULAR ANALYSIS OF THREE STRUCTURALLY SIMILAR BUT FUNCTIONALLY DIFFERENT P_{II}-LIKE SIGNAL TRANSMITTER PROTEINS IN THE DIAZOTROPH *AZOARCUS* SP. BH72

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P_{II}-like signal transmitter proteins are known to be involved in the regulation of ammonia assimilation and nitrogen fixation. Genes encoding P_{II}-like proteins have been identified in several phylogenetically distinct groups of prokaryotes such as *Proteobacteria*, Cyanobacteria, Gram-positives and *Archaea.* Recently, *glnB*-like genes have also been identified in plants, namely *Porphyra purpurea*, a red algae, *Arabidopsis thaliana* and *Ricinius communis.* The presence of two P_{II}-like proteins encoded by *glnB*- and *glnK*- homologues has been described in several species of the *Proteobacteria.*

Here, we report for the first time the identification of three functional P_{II}-like proteins in the N_2-fixing, endophytic bacterium *Azoarcus* sp. BH72, encoded by the *glnB* gene and the *glnK-amtB* and *glnY-amtY* operons, respectively. Phylogenetic analysis carried out with three different algorithms revealed that *glnK* and *glnY* of *Azoarcus* sp. BH72 represent distinct lineages in the phylogenetic tree from which the proteobacterial *glnK* clade descents. The tree topology indicates that *glnK* and *glnY* originated from *glnB* by two early gene duplication events during evolution of the β-subgroup of *Proteobacteria.* Southern blot hybridisation indicated that the occurence of three P_{II} protein-encoding genes is widespread among the genus *Azoarcus.*

A combined approach of two-dimensional gel electrophoresis, Western blotting, N-terminal sequencing and marker exchange mutagenesis allowed to analyse P_{II} protein expression of *Azoarcus* sp. BH72 *in vivo.* In contrast to other proteobacteria, GlnK was the predominant paralogue on all nitrogen sources tested. However, knockout mutant analysis revealed a hierarchic expression of the P_{II}-like proteins (GlnK → GlnB → GlnY), suggesting that regulatory pathways controlling their expression are interconnected. Nitrogen limitation enhanced transcription of *glnK* strongly, *glnY* moderately and *glnB* not at all. Comparison of mRNA abundance and corresponding protein levels revealed that expression of *glnK* and *glnY* is also modulated by differential transcript stability and translational efficiency.

Phenotypic characterisation of knockout mutants elucidated that unlike in other *Proteobacteria*, neither *glnK* nor *glnB* were essential for nitrogen fixation. However, the third paralogue (*glnY*) might fullfil this function in the *glnB/K*-double-mutant. Since the growth of a *glnB/K-double* mutant on minimal media was drastically impaired, both proteins are probably involved in control of ammonia and nitrate assimilation. Despite the structural and some functional similarities, there were differences in details of N-sensing. All three P_{II} proteins were covalently modified by uridylylation upon nitrogen limitation as shown by mass spectrometry, however the modification patterns in relation to the supplied nitrogen source differed, indicating that the three proteins have distinct functions in nitrogen sensing. The novel paralogue GlnY was highly unusual since it only occurred in the uridylylated state *in vivo* and thus lacked a deuridylylation response to nitrogen excess.

Characterization of regulatory cascades and proteins that interact with the different paralogues should provide additional insights into the stimuli and the signalling mechanisms regulating nitrogen metabolism.

ESSENTIAL DNA SEQUENCE FOR *NIFA* EXPRESSION IN *AZOSPIRILLUM BRASILENSE.*

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Azospirullum brasilense is a free-living diazotrophic bacterium found in association with gramineae. The expression of the *nif* genes is activated by the NifA protein under nitrogen fixation conditions. While this process is conserved among the diazotrophs, regulation of *nifA* expression varies considerably. The *nifA* gene of *A. brasilense* is expressed from an unidentified promoter under nitrogen fixation conditions and in the presence of NH^+_4 and oxygen (Liang et al., 1991), but its regulation is not completely understood. In this work we have identified the minimum region essential for *A. brasilense nifA* gene expression, and the regulation of the *nifA* gene was studied in plasmid and chromosomal *nifA::lacZ* fusions.

The structure and activity of the *nifA* promoter of *A. brasilense* was studied by deletion analysis. An 0,6 kb *Sal*I –*Eco*RI fragment of *A. brasilense* DNA containing the putative promoter region of *nifA* was sequentially deleted from the 5' end and subcloned into the fusion vector pPW452. Further deletions were constructed by PCR and cloned in pPW452. The activities of the native and truncated promoters were analysed in *A. brasilense* wild type (FP2) and putatives *ntrC* (FP8 and FP9) and *nifA* (FP10) mutants (Pedrosa, Yates, 1984), and in *E. coli* wild type (ET 8000), *ntrC* (ET 8556) (MacNeil et al., 1982) and *rpoN* (TH1) (Hunt, Magasanik, 1985) mutants. *A. brasilense* chromosomal *nifA::lacZ* fusions were constructed by Tn*5*::B20 (Simon et al., 1989) insertion.

Activities of plasmid *nifA::lacZ* fusions indicated that *nifA* gene expression is independent of NifA, NtrC and σ^N. The promoter activity decreased progressively reaching background levels after the removal of 204 bases from the *Sal*I site. An essential region for *nifA* promoter activity was identified between nucleotides -67 and -47 from the transcription start site. As with the native promoter, all deleted promoters were repressed by 20 mM NH_4Cl in *A. brasilense*, suggesting that the elements for ammonia repression were still present. A sequence resembling a -35/-10 type promoter was also identified and may constitute the *nifA* gene promoter. Expression of the chromosomal *nifA::lacZ* fusion in the *A. brasilense* wild type (FP2) and $NtrC^-$ mutant (FP9), grown under air, was repressed (80-90%) by 20 mM NH_4Cl. Full expression of *nifA* was obtained under low oxygen levels and in the absence of ammonium ions. The results suggested that *nifA* repression is dependent on a synergistic effect of both oxygen and ammonium.

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DIFFERENTIAL EXPRESSION OF *fixNOQP* REITERATED GENES IN *RHIZOBIUM ETLI* BY A NOVEL *fixL*-fixK* CASCADE.

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The elements of the *fix* cluster in *Rhizobium* are well conserved, but the functional and structural organization of these genes is particular for each *Rhizobium* species. In *R. etli* CFN42 reiterations of a fix region, spanning *fixK*, *fixNOQP* and *fixG* are located on both the pSym and pCFN42f.
For both reiterations, the *fixNOQP* and *fixGHI* operons are transcribed in the same direction, divergent to the *fixK* gene. Additionally, pCFN42f show, downstream of *fixK*, an unusual *fixL* (*fixL**) gene. No *fixJ* homologswere detected, searching by heterologous hybridization and degenerate PCR. The presence of this *fixL** and the absence of a conventional *fixJ* is shared with other *R. etli* strains and *R. leguminosarum* bv viciae (VF39).

The contribution of each *fixK* gene and *fixL*-fixK* system on the regulation of their target genes, was analyzed using transcriptional fusions. Our results show that FixK(pCFN42f) controls the expression of both *fixNOQP* homologs, but FixK(pSym) does not exhibit any regulatory role. However, *fixNOQP* homologs are differentially regulated. A mutation in *fixL** blocks the expression of *fixK*(pCFN42f) and *fixNOQP*(pCFN42f), but *fixNOQP*(pSym) is still expressed, suggesting the existence of additional regulatory elements.

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NITROGEN FIXATION GENES CONTIGUOUS WITH THE *nifHDK* GENES OF *HERBASPIRILLUM SEROPEDICAE*

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H. seropedicae is a gram-negative, endophytic bacterium found in association with several grasses, which fixes nitrogen under low oxygen tension and in the absence of fixed nitrogen (Baldani et al., 1986; Klassen et al., 1997). In this work we have isolated and sequenced 10 kb downstream from the *nifHDK* genes and Tn*5*:20 or Km cassette insertional mutations in the region were obtained and characterized.

The 2 kb *Sal*I fragment containing the *nifDK* genes *of H. seropedicae* was used as a probe to screen a λEMBL3 genomic library of *H. seropedicae* by plate hybridization. A recombinant phage, λGK10, was isolated and fragments of its insert were subcloned and sequenced. Downstream from the *nifHDK* genes in a 1.2 kb *Sal*I/*Eco*RI DNA fragment was the N-terminal of *nifE* together with C-terminal of the *nifK* gene. The intergenic region did not reveal any sequence homologous to a *nif* promoter consensus sequence nor to any other known promoter. This result was confirmed by subcloning this region in a *lacZ* fusion vector which revealed no promotor activity in the *nifKnifE* intergenic region. In a contiguous 4.0 kb *Eco*RI DNA fragment we found the C-terminal of the *nifE* gene and *nifNXorf1orf2orf3*. Insertional mutagenesis of *nifN*, *nifX* and *orf*1 showed that *nifN* is essential for nitrogen fixation in *H. seropedicae* but *nifX, orf1, orf2* and *orf3* were not. No promoter was found in the sequenced region; complementation studies and *lacZ* fusion indicated that the *nifHDKENX orf1orf2orf3* genes comprise a single operon regulated by O_2 and NH_4^+. Downstream from this region we found the genes *nifQmodABCfixXC*. *nifQ* and *modA* insertion mutants were also obtained. These genes were essential for nitrogen fixation only under low molybdenum conditions. The *modA* mutant was also deficient in nitrate-dependent growth, probably due to low activity of the molybdoenzyme nitrate reductase. *nifQmodABC* probably comprise a single operon expressed from the *nifQ* promoter since no promoter activity was found downstream from *nifQ*.

The structural organization of *nif* genes observed in *H. seropedicae* has not been found in any other diazotrophic organism. In *Bradyrhizobium japonicum* the *nifDKENX* genes are organized in a single operon with a promoter upstream from the *nifD* gene (Aguilar et al., 1990). The methanogenic archaeon *Methanococcus maripaludis* contains the *nif* operon *nifHorf105orf121nifDKENX* with a *nifH* promoter sequence (Kessler et al., 1998). *H. seropedicae* differs from *Klebsiella pneumoniae* (Merrick, 1992), *Azotobacter vinelandii* (Jacobson et al., 1989) and *Azospirillum brasilense* (Passaglia et al., 1991) in the absence of the *nifTY* genes downstream from *nifHDK*. Furthermore only *Rhodobacter capsulatus* was reported to contain the genes involved in molybdenum transport contiguous with the nitrogen fixation genes (Wang et al., 1993).

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EXPRESSION AND PURIFICATION OF THE GLNB AND GLNZ PROTEINS OF *AZOSPIRILLUM BRASILENSE* STRAIN FP2

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Azospirillum brasilense is a diazotroph found in association with important agricultural crops such as maize, wheat, banana and pineapple (Döbereiner, 1991; Cruz et al., 1999). In this organism, as in other diazotrophs, nitrogen fixation gene expression is under control of fixed nitrogen and oxygen (Döbereiner, Pedrosa, 1987).

The GlnB (PII) protein of *A. brasilense* and its paralogue GlnZ are proteins involved in nitrogen metabolism regulation. Both proteins are uridylylated *in vivo* under conditions of nitrogen limitation (de Zamaroczy et al. 1996); however, although PII is necessary for nitrogen fixation, the role of the GlnZ protein is still unclear.

The *glnB* and *glnZ* genes were amplified from the genome of *A. brasilense* strain FP2 using primers based on the published sequence (de Zamaroczy et al., 1993; de Zamaroczy et al., 1996). The amplified sequences were subcloned into the T7-based expression vectors pT7-7 and/or pET28-a and the recombinant plasmids were used to transform *E. coli* BL21(DE3)pLysS. Upon induction with IPTG or lactose, the transformed cells expressed soluble PII and GlnZ proteins.

The PII protein was expressed either as a fusion to a His-tag sequence or in its native form. The fusion protein PII-HisTag was purified using protamine sulfate precipitation (0.25 mg/mg protein) and affinity chromatography on a **HiTrap-Chelating-Ni^{2+}** resin. The PII-HisTag fusion protein was eluted with 300 mM imidazole. A 97% pure fraction was achieved as revealed by densitometric analysis of an SDS-PAGE. Preliminary assays showed that the protein was uridylylated *in vitro* by a crude extract of *A. brasilense.* A native form of the PII protein was also purified using protamine sulfate precipitation and chromatography on DEAE-Sepharose and Heparin resins, achieving 90% purification.

The GlnZ protein was expressed in its native form. It was purified using protamine sulfate precipitation (0.25 mg/mg protein) and chromatography on Q-Sepharose and Heparin resins. The protein was eluted from both columns with 600 mM KC1. Densitometric analysis of an SDS-PAGE indicated 97% purity.

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EXPRESSION AND RESTORATION OF NIF$^+$ PHENOTYPE TO *AZOTOBACTER VINELANDII* AND *AZOSPIRILLUM BRASILENSE* MUTANTS BY *ACETOBACTER DIAZOTROPHICUS nifA.*

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The ability of NifA to activate *nif* gene expression is ubiquitous to all diazotrophic proteobacteria so far studied. However, the mechanism which elicit *nifA* gene transcription and expression are particular to some groups. *Acetobacter diazotrophicus* is an endophytic diazotrophic bacterium found in high numbers inside sugarcane tissues. The peculiar ability to express nitrogenase in presence of KNO_3 and partially in other N compounds reinforces its potential for BNF contribution to sugarcane crops. The *A. diazotrophicus* whole *nifA* and part of *nifB* were isolated, sequenced (Teixeira et al., 1999) and subcloned into pRK290. Transconjugants of *A. vinellandii* MV521 (*ntrCnifA*) and *A. brasilense* FP10 (*nifA*) carrying pRKNifA were selected based on the antibiotic resistance and Nif+ phenotype. The wild type, mutants and transconjugants strains of each species were grown under appropriate conditions, according to their requirements, for the expression of BNF and nitrogenase activity. The *A. brasilense* were grown in liquid Nfb medium containing 5 mM of sodium glutamate, as source of N. *A. vinelandii* were grown in Burk`s medium containing 1 and 5 mM of ammonium acetate. After 72 hours of growth, cultures were submitted to acetylene reduction assay (ARA) to evaluate nitrogenase activity. The wild type (FP2) and transconjugant FP10 (pRKNIFA) of *A. brasilense* showed nitrogenase activity of approximately 15 nmol.mg of $\text{protein}^{-1}.\text{min}^{-1}$, while the FP10 mutant showed no nitrogenase activity as expected due to mutation of the *nifA* gene. The specific activity of FP10 (pRKNIFA) was comparable with the wild type (FP2) indicating that the *nifA* gene of *A. diazotrophicus* was capable to complement the mutation in *A. brasilense* FP10 and restore up to 100% of its ability to fix nitrogen. The mutant *A. vinelandii* MV521 showed no specific activity for the nitrogenase but the wild type and the transconjugant MV521 (pRKNIFA) showed nitrogenase activity in both treatments. When 1mM of ammonium acetate were added as N-starter dose the wild type (UW136) showed high activity than the transconjugant MV521 (pRKNIFA), but there was no difference in the specific nitrogenase activity with 5mM of N-combined. The results obtained with 1mM of N suggest that the complementation of *A. vinelandii* mutant with the *A. diazotrophicus nifA* gene were not 100% as compared with wild type. However, we cannot discard a problem due to the initial cell numbers since it was observed that the accumulation of the content of proteins did not correspond to the rate of nitrogen fixation between the wild type and the transconjugant.

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STUDIES ON THE ROLES OF GLNK AND PII IN REGULATING *KLEBSIELLA PNEUMONIAE* NIFL-DEPENDENT NITROGEN CONTROL

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In *K. pneumoniae,* the activity of the *nif*-specific transcriptional activator NifA is modulated by NifL which mediates both oxygen and nitrogen control. The signal transduction protein GlnK is required to relieve the inhibitory effect of NifL on NifA that occurs when the intracellular N status is high, and in a wild type cell the action of GlnK cannot be substituted by the structurally related protein PII. We have studied the modulation of NifA activity by NifL in a heterologous system in which the host organism is *E. coli.*

Using a **Δ*glnB*Δ*glnK*** mutant, we have shown that the modulation of NifA activity by NifL is dependent on the concentration of GlnK in the cell and that when overproduced PII can substitute for GlnK. Our data also suggest that the relief of NifL-mediated inhibition may involve both changes in the intracellular level of GlnK and changes in its activity. Furthermore, when we uncouple transcriptional control of *glnK* from the Ntr system we observe a marked antagonistic effect of PII on GlnK that is more pronounced when GlnK is not uridylylated. This effect could be direct or indirect but could conceivably occur by the formation of heterotrimers between PII and GlnK with a consequent inactivation of GlnK. This negative effect of PII may be physiologically important in establishing repression of *nif* gene expression when the intracellular nitrogen status raises.

Effects of GlnK and PII on p*nifH-lacZ*[a] expression in different backgrounds.

	Plasmids											
	-		pCC46 *nifL*$^+$ *nifA*$^+$		pCC47 *nifA*$^+$		pCC46, pWVH149 *nifL*$^+$ *nifA*$^+$ *glnK*$^+$		pCC46, pTA48 *nifL*$^+$ *nifA*$^+$ *glnK*Y51F		pCC46, pAH5 *nifL*$^+$ *nifA*$^+$ *glnB*$^+$	
Strain	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N
wt	10	5	1180	25	4150	4300	3500	810	930	35	1050	140
Δ*glnB*	30	15	2200	15	8500	5990	nd	nd	2750	350	nd	nd
Δ*glnK*	60	25	60	50	6660	6370	1955	420	290	25	800	95
Δ*glnBK*	60	55	50	60	7600	8000	4420	890	2235	360	1180	185

[a] β galactosidase activity (Miller units)
-N: Nitrogen-limiting medium, +N: Nitrogen-sufficient medium
nd = not done

THE *AZOSPIRILLUM BRASILENSE* TWO-COMPONENT REGULATORY SYSTEM COMPOSED BY NTRY AND NTRX PROTEINS IS INVOLVED IN THE REGULATION OF NITROGEN FIXATION.

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The *ntrYX* genes have been previously identified and sequenced in *Azorhizobium caulinodans.* The products of these genes show homology with the two-component regulatory system NtrB/NtrC, and it was suggested that they were involved in control of nitrogen fixation and metabolism in that organism (Pawlowski et al., 1991). These genes *ntrYX* have been were found in two others bacteria: *Acetobacter diazotrophicus* (Meletzus et al., 1998) and *Rickettisia prowazekii* (Anderson et al., 1998). In these organisms, the functions of the proteins NtrY and NtrX are still not understood. In *Azospirillum brasilense,* deletion of *ntrBC* genes did not prevent nitrogen fixation but reduced it to half of that observed in the wild type strain (Machado et al., 1995; Liang et al., 1993). This observation suggested the involvement of another system in the regulation of the expression of nitrogen fixation. We found two ORFs located downstream from the *ntrBC* of *A. brasilense* that showed homology with *ntrY* and *ntrX* genes from *A. caulinodans.* The *A. brasilense* NtrY-like protein showed homology with transmembrane chemoreceptor and sensor proteins while the NtrX protein contained a potential DNA-binding site, suggesting that this protein could act as a regulatory protein.

A *lacZ*-kanamycin cassette (pKOK6.1, Kokotec et al., 1989) was inserted into a *Nsi*I site in the *ntrY* gene cloned in a suicide plasmid, pSUP202. The resulting plasmid, pKL2, was then transferred to wild type *A. brasilense* strain FP2 by electroporation. Several mutants were obtained and one was selected (MLY9) for further characterization. The expression of these genes was studied in *ntrC* and *rpoN* mutants of *Escherichia coli* (ET8556 and TH1, respectively) grown in different ammonium concentrations (2 mM and 20 mM of NH_4Cl).

The mutant MLY9 showed null acetylene reduction activity compared to that of the wild-type FP2 but the Nif^- phenotype was partly complemented by the product of *Klebsiella pneumoniae nif*A gene expressed constitutively. The MLY9 mutant grew poorly on nitrate as the sole nitrogen source but grew well in ammonia and glutamate. The motility of MLY9 was 60% lower than FP2 in nitrogen-limiting conditions although the number of flagella was similar. The expression of glutamine synthetase was 60% lower in MLY9 than that observed in FP2 but the level of GS adenylylation was similar under nitrogen-fixing conditions. These results suggest that the NtrY/NtrX proteins play a role in the regulation of nitrogen fixation, growth on nitrate, motility and are necessary for maximum expression of glutamine synthetase, but are not involved in the regulation of GS activity by adenylylation in *A. brasilense.*

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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF THE CHROMOSOMAL *NIFA* REGION OF *ACETOBACTER DIAZOTROPHICUS*

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The nifA gene of Acetobacter diazotrophicus was isolated by complementation of *nifA* mutants of *Azotobacter vinelandii.* Mutants carrying *gusA*-kan interposons reveal, that *nifA* expression is not influenced by the cellular oxygen level, but is modulated in response to different levels of fixed nitrogen (Teixeira et al. 1999). However, so far we have not been able to identify characteristic promoter elements known from nitrogen regulated genes of other diazotrophs. Furthermore *ntrC* mutants do not have an influence on expression of the *nifA* gene.

In order to obtain more information on adjacent gene loci, the DNA sequence of the chromosomal *nifA* region was analysed. Our data indicate that the *nifB* gene downstream of *nifA* is part of a *nifB-fdxN-ynfI-nifZ-fixU* operon. In addition we were able to identify the *modABC* genes and the *rpoN* gene upstream and opposite to *nifA*. Interestingly no *modE-like* gene is located within this chromosomal region and a *modD-like* gene locus has been found elsewere on the chromosome (C. Kennedy, personal communication).

Work is in progress to identify the regulatory genes involved in *nifA* expression and the functional analysis of the adjacent genes.

Teixeira KRS et al (1999) FEMS Microbiological Letters 176, 301-309

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PURIFICATION AND BINDING ANALYSIS OF NIFA PROTEIN FROM *AZOSPIRILLUM BRASILENSE.*

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NifA of *A. brasilense* was purified in the form of a glutatione-S-transferase (GST)-NifA fusion protein and proteolytic release of GST yielded inactive and partially soluble NifA. However, the purified NifA was able to induce the production of specific anti-*A brasilense* NifA-antiserum that recognize NifA from *A. brasilense* but not from *K. pneumoniae*. Both GST-NifA and NifA expressed from *E. coli tac* promoter are able to activate transcription from the *nifHDK* promoter but only in *A. brasilense* background. *E.coli* total protein extracts have been used to analyse the NifA in mobility shift assays. DNA fragments carrying the two overlapping, wild-type or mutated, UAS motifs present in the *nifH* promoter region revealed a retarded band of related size. The binding activity present in the C-terminal domain of *A. brasilense* NifA protein is still functional even in the presence of oxygen.

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THE EXPRESSION OF *nifB* GENE FROM *HERBASPIRILLUM SEROPEDICAE* IS DEPENDENT UPON THE NIFA AND RPON PROTEINS

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Herbaspirillum seropedicae is a microaerophilic gram-negative nitrogen-fixing bacterium which occurs in association with gramineae. It is a free-living diazotroph under laboratory conditions, vibrioid, sometimes helical and very motile (Baldani et al., 1986).

The *nifB* gene of *Herbaspirillum seropedicae* was sequenced and the deduced amino sequence of the NifB protein showed 62, 57 and 47% identity with the NifB proteins of *Bradyrhizobium japonicum, Rhodobacter capsulatus and Klebsiella pneumoniae,* respectively (Souza et al., 1991). The putative *nifB* promoter region of *H. seropedicae* containing two sequences homologous to a NifA UAS , a -24/-12 type promoter and a possible IHF-binding motif. To determine the involvement of NifA and σ^N (the product of the *rpoN* gene) in the activation of the *nifB* promoter of *H. seropedicae,* we studied *nifB* expression *in vivo* and DNA band shift experiments *in vitro.*

A *nifB::lacZ* transcriptional fusion was constructed and analyzed in NtrC or RpoN mutant strains of *E. coli* in the presence or absence of *K. pneumoniae* NifA, expressed constitutively from pMC71A. *H. seropedicae* wild type and NifA mutant strains containing chromosomal and plasmid fusions were also analyzed. In *E. coli,* the *nifB* promoter of *H. seropedicae* was activated only in the presence of both σ^N and the *K. pneumoniae* NifA protein. In the *H. seropedicae* wild type containing a plasmidial or chromosomal fusion the *nifB* gene was expressed only in the absence of ammonia and at low levels of oxygen; in the NifA^- mutant, *nifB* was not expressed under any condition tested.

Furthermore, DNA band shift experiments showed that the *K. pneumoniae* NifA protein, purified RpoN and *E. coli* IHF protein were capable of specific binding to the *nifB* promoter region of *H. seropedicae.* These results strongly suggest that the expression of the *nifB* promoter of *H. seropedicae* is dependent on the NifA and σ^N proteins.

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PHYSIOLOGICAL CHARACTERIZATION OF THE OPERON *glnAntrBC* OF *HERBASPIRILLUM SEROPEDICAE*

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The operon *glnAntrBC* of *Herbaspirillum seropedicae* has been sequenced and the upstream region of the *glnA* gene revealed one potential –24/-12 promoter and two putative NtrC-binding sites, which overlap a sequence similar to a canonical σ^{70} promoter, suggesting that the expression of this operon is under nitrogen status control. No obvious promoter sequence was observed between *glnA* and *ntrB.*

The *ntrC* gene has been mutated by Tn5-B20 insertion yielding strain DCP286A. This mutant failed to grow on nitrate as the nitrogen source and was Nif^-. Both phenotypes were complemented by a plasmid carrying the operon *glnAntrBC.*

The β-galactosidase activity of the wild type carrying a *glnA::lacZ* fusion was regulated by ammonia but in the *ntrC* mutant it was not. The total GS activity (total transferase activity) in the wild type increased 2-3 fold when assayed under nitrogen limitation (5 mmol/L glutamate and 2 mmol/L NH_4Cl) whereas in the *ntrC* mutant strain these levels remained unchanged. The GS biosynthetic activity, was low in the *ntrC* mutant irrespective of the nitrogen levels. These results suggest that NtrC activates and regulates the expression of *glnAntrBC* operon. To verify the effect of the *ntrC* mutation on the adenylylation of glutamine synthetase, cells of the strains SMR1 (wild type) and DCP286A (*ntrC* mutant) grown in NFbHP containing NH_4Cl 20 mmol/L were collected and resuspended in N-free NFbHP. Initial levels of glutamine synthetase activity were similar, but after incubation in the absence of ammonia the wild type showed an increase of GS biosynthetic activity which decreased to the initial level with addition of NH_4Cl (20 mmol/L) . In contrast, GS activity remained consistently low in the mutant. The effect was repeated in the presence of tetracycline or chloramphenicol, confirming that there is no interference caused by *de novo* protein synthesis. These results suggest that NtrC is involved in the regulation of GS synthesis and activity control.

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PARTIAL CLONING OF THE AMMONIUM TRANSPORTER GENES OF *AZOSPIRILLUM BRASILENSE* AND *HERBASPIRILLUM SEROPEDICAE.*

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Azospirillum brasilense and *Herbaspirillum seropedicae* are diazotrophs found in association with important agricultural crops as maize, wheat, banana, pineapple, sugar cane and rice (Döbereiner, 1991; Cruz et al., 1999; Baldani et al., 1986). Nitrogen fixation is regulated by ammonium and oxygen in both microorganims (Döbereiner, Pedrosa, 1987).

Ammonium transporter genes (*ami*) have been described in several organisms and they code for transmembrane proteins with high homology (Merrick, Edwards, 1995). High ammonium concentrations seem to inhibit this transport and, in some organisms, the *ntr* system is involved (Merrick, Edwards, 1995). Here we describe the partial cloning of the ammonium transporter gene (*amtB*) of *A. brasilense* and *H. seropedicae.*
Degenerate primers related to homologous aminoacid sequences of the ammonium transporter proteins of *E. coli, B. subtilis* and *M. tuberculosis* were used to amplify DNA fragments of *A. brasilense* strain FP2 and *H. seropedicae* strain SmR1. These amplified DNA fragments were cloned and sequenced. A 150 bp DNA fragment amplified from the genome of *H. seropedicae* showed 68% identity with the *E. coli* AmtB at the aminoacid level and to other ammonium transporter proteins present in the database. This result indicates the presence of an *amtB*-like gene in *H. seropedicae.* A 510 bp DNA fragment amplified from the genome of *A. brasilense* strain FP2 was almost identical to the sequence published by Van Dommelen et al (1998) except for 2 positions (GenBank AF082303). This DNA fragment was used to generate an *amtB*$^-$ mutant of *A. brasilense* by insertion of a *lacZ::Km* cassette. This mutant showed a decrease in ammonium uptake of 43% compared with the wild type when tested at pH 6.8 and when grown in 5 mM glutamate. No significant difference was observed when cells were grown in 20 mM NH_4Cl, suggesting that the *amtB* gene is expressed under ammonium-limiting conditions. In both the wild type and the mutant strains, a decrease of about 50% in ammonium uptake was observed when the pH was decreased from 6.8 to 5.6. This effect of pH on ammonium uptake suggests, as proposed by Soupene et al (1998), that NH_3 is the transported species.

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FUNCTIONAL DIFFERENCE BETWEEN THE PII PROTEINS FROM *HERBASPIRILLUM SEROPEDICAE* AND *KLEBSIELLA PNEUMONIAE*

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Herbaspirillum seropedicae is an endophytic diazotroph isolated from grasses. In this microorganism, nitrogen fixation expression is regulated by the NifA protein. The expression of the *nifA* gene is under control of the Ntr system, the general regulator of nitrogen metabolism. The PII protein, coded by the *glnB* gene, has a central role in control of the Ntr system. X-ray data suggest that the PII protein of *H. seropedicae* has a similar structure to the GlnK protein of *Escherichia coli* (Xu et al., 1998). The major difference between PII proteins of *H. seropedicae* and *E. coli* was found in the C-terminal region which contains a 3_{10}-helix that is not present in *E. coli* PII. However, a similar structural motif occurs in the *E. coli* GlnK protein, a paralogue of PII. The PII of *H. seropedicae* is essential for NifA activity control, raising the possibility that the C-terminal region of *H. seropedicae* PII signals the nitrogen levels to NifA (Benelli, personal communication).

The plasmids pEMB101, containing the *glnB* gene from *H. seropedicae,* and pACB100, containing the *glnB* gene from *Klebsiella pneumoniae,* were introduced into *Azospirillum brasilense* strain FP9, which is Nif minus and expresses very low levels of PII (de Zamaroczy et al., 1993). The *H. seropedicae glnB* was able to complement the Nif minus phenotype of FP9, whereas the *glnB* of the enteric bacterium *K. pneumoniae* produced very low levels of nitrogenase activity. This parallels the role of GlnK protein in enteric diazotrophs which controls the activity of the NifL protein (He et al., 1998; Jack et al., 1999).

To confirm the importance of the C-terminal region of the PII protein from *H. seropedicae* site directed mutants have been obtained. The sequences that code for the amino acid residues Gly108 and Pro 109 were substituted by sequences that code for two alanines. This mutant was named PII-AA. In addition, the mutant PII-AL (Q3R, T5A, G87C e G108W) was obtained by random mutagenesis. The mutants will be used to study interactions of the PII protein with GlnD, GlnE and NtrB proteins both *in vivo* and *in vitro.* The plasmids pACB200, containing the *glnB* gene from *H. seropedicae,* pACB2, containing the PII-AA and, pACB50, containing the PII-AL were introduced into *K. pneumoniae* strain UNF1529 which is Nif minus. These transconjugants were able to complement nitrate growth, but were unable to complement the Nif minus phenotype of UNF1529.

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AMMONIUM AND MOLYBDENUM REGULATION OF THE MOLYBDENUM NITROGENASE AND THE ALTERNATIVE NITROGENASE IN *RHODOBACTER CAPSULATUS*

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The phototrophic purple bacterium *R. capsulatus* is able to fix atmospheric dinitrogen either via a conventional molybdenum nitrogenase or via an alternative iron-only nitrogenase. Synthesis and activity of both nitrogenase systems is tightly regulated by a number of environmental signals including the availability of ammonium and molybdenum. Ammonium control is mediated by a nitrogen regulation (Ntr) system similar to that of enteric bacteria. The *R. capsulatus* Ntr system consists of the histidine kinase NtrB, the response regulator NtrC, and the signal transducer GlnB. As many other bacteria, *R. capsulatus* contains a second *glnB*-like gene (*glnK*), which is closely linked to *amtB* coding for a putative (methyl)-ammonium transporter. In contrast to all other known members of the NtrC family, *R. capsulatus* NtrC activates transcription in concert with RNA polymerase containing the house-keeping sigma factor σ^{70} instead of σ^{54} (Bowman *et al.* 1998). Targets of *R. capsulatus* NtrC comprise *nifA1, nifA2,* and *anfA* (coding for the transcriptional activators of the molybdenum nitrogenase and the heterometal-free nitrogenase) as well as *glnB* and *glnK.* In addition to NtrC dependent activation, *anfA* transcription is repressed by traces of molybdenum via the two molybdate dependent repressor proteins MopA and MopB (Wang *et al.* 1993).

Mutations in *glnB* resulted in constitutive expression of *nifA1, nifA2,* and *anfA.* In contrast, transcriptional regulation of *nifA* and *anfA* was unaffected by mutations in *glnK.* However, expression of both nitrogenase systems was still negatively regulated by ammonium indicating the presence of an NtrC- and GlnB-independent posttranslational mechanism controlling the activity of NifA1, NifA2, and AnfA. These findings were corroborated by analyses of *R. capsulatus* mutant strains constitutively expressing either *nifA1* (Hübner *et al.* 1993), *nifA2* or *anfA.* In addition to ammonium regulation, AnfA activity was controlled by molybdenum at the posttranslational level in a MopA/B-independent manner, whereas a *mopA-mopB* double mutation in a *nifHDK* mutant background led to complete loss of Mo control. To test whether *glnB* and *glnK* can substitute for each other in ammonium regulation of the activity of NifA and/or AnfA, a *glnB* mutant strain was constructed, which carries a *glnK* plasmid-integration mutation. The resulting truncated GlnK protein lacks the C-terminal 19 amino acid residues. In this mutant background NifA1 and NifA2 were no longer subject of posttranslational ammonium control, whereas AnfA activity was still repressed by ammonium. Unexpectedly, Western blot analyses revealed the loss of ADP ribosylation of both nitrogenase reductases (NifH, AnfH), indicating an impairment of DraT/G activity. Furthermore, in vivo activity of the molybdenum nitrogenase was not repressed by ammonium any more, suggesting circumvention of all known negative control mechanisms in a *glnB-glnK* double mutant.

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GENETIC CHARACTERIZATION OF Nif CONSTITUTIVE MUTANTS OF *Azospirullum brasilense*

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Azospirillum brasilense, a Gram-negative diazotrophic bacterium, belongs to the α subgroup of proteobacteria and fixes nitrogen only under microaerophilic conditions in the absence of ammonium ions. Nitrogenase activity is not observed in the presence of high NH_4^+ concentrations, in *A. brasilense* as reported for other diazotrophs (Postgate, 1982). A number of regulatory mutants of *A. brasilense* have been isolated (Pedrosa and Yates, 1984 ; Fischer et al., 1986; Machado et al., 1991) and these mutants are important for understanding the regulation of *nif* gene expression in this diazotroph. Enterobacterial mutants defective in the nitrogen control regulatory system (Ntr) generally display a pleiotropic phenotype with regard to expression and regulation of several enzymes and transport systems involved in the assimilation of N sources.

Four spontaneous mutants HM14, HM26, HM053 and HM210 of *A. brasilense* strain FP2 resistent to ethylenediamine were isolated and partially characterized (Machado et al., 1991). The mutants fixed nitrogen constitutively in the presence of high NH_4^+ concentrations, but were defective in nitrate-dependent growth and excreted NH_4^+ derived from nitrogen fixation. The mutants showed glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activities similar to those of the wild type. However, different patterns of GS transferase and adenylylation were observed which allowed their distribution into three classes: the first includes HM053, which showed low levels of constitutively adenylylated GS activity; the second included HM14 which had low levels of regulated GS activity, compared with those of the wild type FP2; the third class, HM26 and HM210, showed wild-type levels of constitutively adenylylated GS activity.

Plasmids carrying *ntr* and *gln* genes were transferred to these mutants by conjugation in an attempt to characterize them genetically through changes in the N-regulation of nitrogen fixation and GS activity. The plasmid pAB441 (*A. brasilense glnBA*) complemented mutants HM26, HM053 and HM210 for nitrate-dependent growth, but repressed nitrogenase under all conditions tested.The plasmid pAB441 restored GS activity and GS regulation by ammonium to strains HM26, HM053 and HM210. The regulation of the wild type FP2 and the HM14 mutant was unaffected by the presence of the plasmid pAB441. The HM14 mutant when conjugated with plasmid pL46 (*A. brasilense ntrYX*) grew on nitrate and the regulation of nitrogenase activity by ammonium ions was restored.

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FUNCTIONS OF THE σ^{54} REGION I *IN TRANS* AND IMPLICATIONS FOR TRANSCRIPTION ACTIVATION

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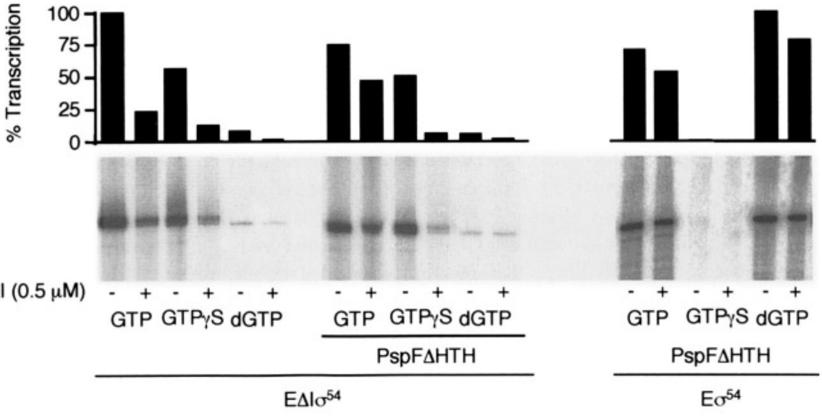
Control of transcription frequently involves the direct interaction of activators with RNA polymerase. In bacteria, formation of stable open promoter complexes by the sigma-54 RNA polymerase depends critically upon sigma-54 amino Region I sequences. Their presence correlates with activator dependence and removal allows the holoenzyme to engage productively with melted DNA independently of activator. Using purified Region I sequences and holoenzymes containing full length ($E\sigma^{54}$) or Region I deleted sigma-54 ($E\Delta I\sigma^{54}$), we have explored the involvement of Region I in transcription activation. Results show that Region I *in trans* inhibits a reversible conformational change in the holoenzyme believed to be polymerase isomerisation. Evidence is presented indicating that the holoenzyme (and not the promoter DNA *per se*) is one interacting target used by Region I in preventing polymerase isomerisation. Activator overcomes this inhibition in a reaction requiring nucleotide hydrolysis. Region I *in trans* is able to inhibit activated transcription by holoenzyme containing the full length sigma-54. Inhibition appeared non-competitive with respect to activator, suggesting that a direct activator interaction occurs with parts of holoenzyme outside Region I. Stabilisation of isomerised holoenzyme bound to melted DNA by Region I *in trans* occurs largely independently of initiating nucleotide, suggesting a role for Region I in maintaining the open complex.



Overcoming of Region I inhibition by activator.

σ^{54}- and $\Delta I\sigma^{54}$-holoenzymes (100 nM) with 4 mM GTP, GTPγS or dGTP and 4 μM activator (PspFΔHTH) were incubated with template DNA (10 nM, pMKC28) prior to heparin challenge and the addition of remaining NTPs plus [α-^{32}P]UTP. GTP, GTPγS and dGTP were used for hydrolysis by the activator and initiation of transcription, only initiation or only activator hydrolysis, respectively. Region I (RI, 0.5 μM) was added prior to holoenzyme assembly.

RPON-DEPENDENT TRANSCRIPTIONAL ACTIVATORS AND THE *RPON* OPERON OF A NITROGEN-FIXING *PSEUDOMONAS STUTZERI* STRAIN.

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The *P stutzeri* strain A1501 (formerly *Alcaligenes faecalis*) can fix nitrogen under microaerobic conditions and colonize rice. In preliminary investigations with strain A1501 it was first checked that nitrogenase activity was observed only in media when the nitrogen source was exhausted and that a *nifH-lacZ* fusion from *A. brasilense* when introduced in the A1501 strain was expressed only under ammonia deficiency. This suggested the existence of functional homologues of *rpoN* and *nifA* genes in A1501.

A gene bank of total DNA was constructed in the broad host range vector pLA29-17. Screening of the bank by colony hybridization and genetic complementation of *Azospirillum* mutant strains led to the identification of several DNA regions sharing similarity with two component systems of the family of **σ54-dependent** activators including *dctBD* and *ntrBC,* as well as a *nifA*-like gene. Genes were identified by nucleotide sequencing and their role was studied by insertional mutagenesis, using classical suicide vehicles. Mutants in the three regions were Nif positive. The *ntrC* mutant is impaired in nitrate and histidine utilization, while the *dctD* mutant was partly impaired in dicarboxylic acid utilization. To further isolate new genes belonging to **σ54-dependent** activators, we have used the degenerated oligonucleotides, designed by Kaufman and Nixon (J. Bacteriol 1996, 178, 3967). Several clones are under study.

It was possible to identify several putative *rpoN* clones, in the gene bank of 1501, by genetic complementation of a *rpoN* mutant of *Azospirillum brasilense* for swarming in semi-solid medium. The nucleotide sequence of one of them revealed the presence of the *rpoN* operon. Inactivation of *rpoN* led to a mutant strain with a Nif minus phenotype, that is also impaired in motility, histidine and nitrate utilization and too a lesser extent in dicarboxylic acid utilization. ORFs downstream from *rpoN* shared high identity with ORF2, *ptsN,* ORF4 and *ptsO* that are commonly found in other genera. Inactivation of these ORFs led to a Nif positive phenotype, although nitrogenase activity of the *ptsO* mutant strain was decreased.

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NIFL OF *Klebsiella pneumoniae:* REDOX CHARACTERIZATION IN RELATION TO THE NITROGEN SOURCE

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In the diazotroph *Klebsiella pneumoniae* products of the nitrogen fixation *nifLA* operon regulate transcription of the other *nif* operons. NifA activates transcription by the alternative form of RNA-polymerase, σ^{54}-holoenzyme. NifL modulates the activity of the transcriptional activator NifA in response to combined nitrogen or external molecular oxygen. We recently showed that *K. pneumoniae* NifL is a flavoprotein which apparently senses oxygen through a redox-sensitive, conformational change (Schmitz, 1997).

K. pneumoniae NifL modulates NifA activity in *Escherichia coli* in response to the level of combined nitrogen, suggesting that NifL might respond to a generalized or globally sensing nitrogen signal transduction pathway. In order to study if the nitrogen signal might be transmitted to NifA through a stable modification of NifL we characterized the redox properties of NifL synthesized in *E. coli* in the presence of different nitrogen sources. FAD analyses showed that purified NifL carried FAD as cofactor independent of nitrogen and oxygen availability. The redox potential of NifL synthesized in the presence of ammonium was -277 ±5 mV at pH 8.0 and 25° C, as determined by reduction with dithionite or with enzymatic reduction by xanthine oxidase in the presence of methyl viologen as redox mediator. When synthesized under nitrogen-limiting conditions, NifL showed a redox potential of- 274 ±6 mV at pH 8.0 and 25° C. Fully reduced NifL fractions, synthesized under either condition listed above, reoxidized rapidly in the presence of molecular oxygen. These results indicate that for NifL synthesized in *E. coli,* the redox potential of the NifL-bound FAD is not influenced by the nitrogen source. The two NifL fractions differed, however, in that a non-flavin specific absorbance at 420 nm was found only in NifL synthesized in the presence of ammonium. One possible explanation for the absorbance at 420 nm is a post translational covalent modification of NifL in response to ammonium: such a modification might yield in an inhibitory form of NifL with unchanged redox potential (Klopprogge, Schmitz, 1999).

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TRANSCRIPTION MEDIATED BY ANFA AND VNFA OF *AZOTOBACTER VINELANDII* RESPONDS TO MO AND V AVAILABILITY

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Azotobacter vinelandii synthesises three genetically distinct nitrogenases. The preferred enzyme contains Mo and, when Mo is not present in the medium, a second enzyme containing V is synthesised in its place. If neither metal is available, a third iron-only nitrogenase is expressed. Transcription of all three nitrogenase systems is driven by sigma54-dependent transcriptional activators, such as VnfA and AnfA, which control expression of the vanadium nitrogenase and the iron-only nitrogenase respectively.

We have investigated metal control of expression of the second and third nitrogenase systems, using *lacZ* fusions both to *vnfA* and *anfA,* and to *vnfH and anfH,* which encode the corresponding dinitrogenase reductases. Expression of the activator genes *vnfA* and *anfA* is modulated by Mo and V to some extent (Premakumar et al., 1998). We eliminated this tier of the metal response for the Anf system by integrating into the chromosome a plasmid carrying the 3' end of *anfA* cloned next to the *tac* promoter. Because the plasmid also carried *lacI,* this subjects *anfA* transcription to *lac* control. Expression of *anfA-lacZ* in such a construct is induced 5-10 fold by IPTG but is unaffected by the presence of Mo and V. Metal control of *vnfA* expression was eliminated by a similar procedure.

In these constructs, little transcription of the nitrogenase structural genes *vnfH* and *anfH* occurs without IPTG. When activator synthesis is induced, the resulting transcription of *vnfH* and *anfH* is still metal modulated, which demonstrates a second tier of metal control. Mo inhibits transcription of both systems, while V inhibits transcription of *anfH-lacZ,* but not *vnfH-lacZ.* Inhibition could be due to metal-sensitivity of the activators themselves because the regulatory domains of VnfA and AnfA may bind metals. However, this may not apply to regulation by Mo because, when *vnfA* and *anfA* are introduced into *Klebsiella pneumoniae* along with the relevant reporter fusions, Mo concentrations 1000-fold higher than those which block transcription in *A. vinelandii* do not affect transcription in the enteric background. Thus, factor(s) peculiar to *A. vinelandii* may be involved in Mo regulation of VnfA and AnfA function. These could be signal transduction proteins interacting directly with the activators, or metal-sensitive repressors binding to the *vnfH* or *anfH* promoters. One possible repressor is ModE, which binds molybdate and can repress transcription from the *anfA* promoter (Premakumar et al, 1998). A mutation in *modE* did not affect Mo regulation of *anfH-lacZ* activity, so another Mo-sensing mechanism in *A. vinelandii* controls transcription of the structural genes for the alternative nitrogenases.

We measured the Mo concentration thresholds at which the ModE-dependent control of *anfA* transcription and the ModE-independent control of *anfH* transcription are switched and found them both to be about 5 nM. Thus, no concentration hierarchy exists in this two-tier system, and there may be a single sensor at the origin or a forked signal transduction pathway. Control of AnfA activity presumably provides a more rapid response to Mo availability than control of its synthesis, but a further selective pressure for this apparently duplicated control may be that the concatenation of two relatively insensitive switches produces a much more clear-cut response than either could alone (Brown et al., 1997).

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DUAL ROLE FOR THE INTEGRATION HOST FACTOR AT AN ENHANCER-DEPENDENT *nifA* PROMOTER

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Control of transcription in σ^N-RNA polymerase-dependent promoters involves direct contact of the regulatory proteins, bound to upstream activator sites (UAS), with RNA polymerase at the promoter (Kustu et al. 1989). This contact is stimulated by the integration host factor (IHF) which binds to a site between the UAS and the promoter to bend the DNA and increase the concentration of activator at the promoter site. This process increases the specific interaction between the σ^N- RNA polymerase and the activator to minimize non-specific contact between the holoenzyme and other activators (Dworkin et al. 1998).

The σ^N-dependent *nifA* promoter of *Herbaspirillum seropedicae (Hs)* is activated under nitrogen-limiting growth conditions. Potential enhancer sites for NtrC and NifA and single binding sites for the IHF and σ^N-RNA polymerase were identified by sequencing, and the RNA start mapped to a G at position 641 (Souza et al 1991; Pedrosa et al. 1997). We have now, by Dnasel footprinting identified two binding sites for NtrC, 3 binding sites for NifA and single sites for the IHF and σ^N-RNA polymerase. Data also shows that NifA and IHF do not mutually restrict each other binding to a linear template. It suggests that the IHF inhibition of NifA-dependent transcription is not due to competition, but to an as yet unknown mechanism.

To determine the contributions of NtrC, NifA and the IHF to transcription from the *nifA* promoter we conducted *in vitro* transcription activation assays in a purified system where the *Hs nifA* promoter was cloned into the transcription vector pTE103, yielding pERW1. Assays involved the binding of the σ^N-RNA polymerase to promoter DNA, followed by the addition of either NtrC or NifA and ATP or GTP to allow open complexes to form. Subsequently heparin and the remaining nucleotides where added to disrupt residual closed complexes and and allow formation of a full length transcript from a heparin-stable open complex. Activation of the *Hs nifA* promoter was shown to be dependent on the σ^N-RNA polymerase, ATP or GTP and the presence of NtrC or NifA. NtrC-dependent activation was stimulated by the IHF with either ATP or GTP. However, transcript formation with GTP was five fold higher than with ATP, whereas the nucleotides were equally effective in activation by NifA. Possibly incorrectly initiated unstable complexes form with NtrC and ATP that do not form with NtrC and GTP or with NifA and ATP. In marked contrast to the IHF stimulation of NtrC-dependent activation, IHF greatly reduced activation by NifA. Presumably, this diminishes potentially destabilizing auto-activation of the *nifA* promoter by NifA. In addition, high levels of NifA depress NtrC-dependent activation.

We conclude that the *nifA* promoter is unlikely to be strongly auto-activated by NifA in cells with IHF when GTP is available. Furthermore, no evidence for synergistic activation between NtrC and NifA was obtained. The different effects of ATP and GTP on NtrC-dependent open complex

NODULE SPECIFIC MODULATION OF GLUTAMINE SYNTHETASE IN TRANSGENIC *MEDICAGO TRUNCATULA*

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The ammonium produced by nitrogen fixation is assimilated in legume nodules primarily by the plant glutamine synthetase (GS). This enzyme is therefore in a key position to play a regulatory role in nodule metabolism and perhaps in the productivity of the plant. In higher plants, GS exists as a number of isoenzymes in the cytosol and plastids and their abundance alters in different tissues and organs of the plant. The heterogeneity of the isoenzymes seems to be under direct gene control, since several genes encode GS and these are differentially expressed during the growth and development of the plant and by different environmental factors. Our studies have shown that *Medicago truncatula* contains one of the smallest plant GS gene families identified to date, having only two cytosolic expressed genes *(MtGSa* and *MtGSb)* (Stanford et al., 1993) and a single gene for plastid-located GS (P. Melo, H. Carvalho and J. Cullimore, unpublished). In *M. truncatula* nodules cytosolic GS makes up over 70% of the GS activity due largely to the expression of *MtGSa* (Carvalho et al., 1997). Immunogold labelling has shown that much of this cytosolic GS is located in the infected cells (Carvalho et al., 1999), where most of the ammonium is believed to be assimilated.

To investigate whether changes in the activity of plant nodule GS alter the nitrogen fixation efficiency and the growth of the plant, we have attempted to modify the expression of the genes *MtGSa* and *MtGSb* in the infected cells of *M. truncatula* nodules, using "sense" and "antisense" technology. For this work we have used the promoter of the leghemoglobin gene *MtLb1* (Gallusci et al., 1991), which expresses only in the infected cells of the central tissues of the nodule. In this way the nitrogen metabolism in other tissues and organs of the plant would not be affected. This promoter was fused to *MtGSa* or *MtGSb* cDNAs, in "sense" or "antisense" orientation, together with the nopaline synthetase (*nos*) terminator. The analysis of enzyme activity has shown a broad variation of GS activity levels in transgenic sense and antisense *M. truncatula* plants. From 14 antisense plants, 2 clearly have decreased GS activity levels when compared to the wildtype. One transgenic sense plant, shows increased levels of GS activity. The data obtained by activity measurements are correlated with the amounts of GS protein, detected by Western blotting.

The altered GS expression present in some of the above plants might influence the overall nitrogen metabolism of the plant. For this reason, further analyses will be performed, to study the pathway of ammonium assimilation in the transgenic plants and the effect of the GS activity modifications on the rate of nitrogen fixation and the productivity of the plant. These studies will provide the means to evaluate the role of GS in controlling flux through the nitrogen assimilation pathway.

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IDENTIFICATION AND CHARACTERIZATION OF GENES INVOLVED IN THE AMMONIUM SENSING MECHANISM IN *ACETOBACTER DIAZOTROPHICUS*

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The PII protein encoded by the *glnB* gene and the uridylyltransferase, product of the *glnD* gene, play a major role in the ammonia sensing mechanism of bacteria.

The *glnB* , *glnZ* and *glnD* genes of *A. diazotrophicus* were isolated by PCR using degenerate nucleotide primers or by complementation of the *nfrX* (*glnD*) mutant strain MV17 of *A. vinelandii* with the pLAFR3 cosmid library. DNA sequence analysis revealed a *glnA* gene downstream of *glnB* and an *amtB-like* gene adjacent to *glnZ,* while the *glnD* gene is cotranscribed with a *mutS*-like gene.

While *glnZ* mutants are not impaired in N_2-fixation, *glnB* mutants are apparently lethal in *A. diazotrophicus.* Preliminary results indicate that the *glnZ* gene product might be involved in posttranslational modification of dinitrogenase reductase. In addition, *glnD* mutants exhibit a severe reduction of the growth rate and are unable to fix nitrogen.

Work is in progress on the molecular analysis of the regulation of these genes and the postranslational modification of the corresponding gene products.

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FUNCTIONAL ANALYSIS OF *nifHDK* OPERON OF *Herbaspirillum seropedicae.*

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1. Introduction

Two contiguous 2.0 and 2.4 kb *Sal*I fragments from the λIM02 and λIM03 phages isolated from a genomic λEMBL3 library of *H. seropedicae* wild type SMR1, were sequenced. Analysis of sequencing data showed the presence of two complete and one incomplete open reading frames (ORFs). These ORFs were identified as *nifH, nifD* and *nifK* genes respectively. These genes showed high homology to the related proteins from several microorganisms such as *Bradyrhizobium japonicum, Azospirillum brasilense* and *Thiobacillus ferrooxidans* (Machado et al.,1996). A sequence similar to that of NifA-activated, RpoN (σ^N)-dependent promoters was found upstream from the *nifH* gene (5'-gTGGgACGGCATTTGCA-3') at -183 bp upstream from the start codon taking the adenine as position 1. Two NifA-upstream activating sequences (5'-TGT-N_{10}-ACA-3') were found at positions -311 and -281 bp from the start codon. In addition, a potential Integration Host Factor (IHF) binding sequence (5'-AATCAAtggcTTG-3') was also found at position -213 from the start codon. Potential ribosome-binding sites are present 6 bp, 3 bp and 7 bp from the start codons of nifH, *nifD* and *nifK*, respectively.

2. Material and Methods

In order to determine the functionality of potential promoters located upstream of *nifHDK* genes, several transcriptional *lacZ*-fusions were constructed in the vector pMP220 or pPW452 (Spaink et al., 1987; P. Woodley) and analyzed in *E. coli* wild type (ET8000) and *rpoN*$^-$ (TH1) strains, containing also the *Klebsiella pneumoniae nifA* gene expressed constitutively. These analyses were carried out in both low (2 mM) and high (20 mM) NH_4Cl concentrations. Complementation studies with a Nif$^-$ (*nifH*::*lacZ*-Km) mutant of *H. seropedicae* with plasmids containing the *nifH*, *nifD* and *nifK* genes were made.

3. Results and Discussion

No expression of β-galactosidase activity was observed in the *rpoN*$^-$ strain, indicating that the promoter located upstream from the *nifH* gene is dependent upon the σ^N factor. A Nif$^-$ (*nifH::lacZ*-Km) mutant *of H. seropedicae* was complemented only by plasmids bearing all the three *nifHDK* genes, suggesting that these genes are organized in a unique operon in *H. seropedicae,* since the mutation has a polar effect on the expression of the genes located downstream. Analysis of *nifH-, nifD-* and *nifK*::*lacZ* transcriptional fusions also support this idea since the *nifHDK* operon was only expressed through a *nifA*-dependent promoter located upstream from the *nifH gene.*

4. References

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5. Acknowledgements

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ROLE OF PII-LIKE PROTEINS IN NITROGEN SENSING BY *AZOTOBACTER VINELANDII* NIFL AND NIFA

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The *Azotobacter vinelandii* NifL-NifA regulatory system is responsive to the redox and fixed N status in *Escherichia coli.* We have used defined nitrogen regulatory mutants of *E. coli* to examine the role of the *glnB* and *glnK* gene products in regulating the inhibitory function of NifL. In contrast to recent results reported for *Klebsiella pneumoniae* NifL, neither *glnK* nor *ntrC* mutations influence the activity of NifL under N-limiting conditions. However under conditions of N-excess our *in vivo* experiments suggest that either GlnB or GlnK can potentiate the inhibitory function of NifL. *In vitro* experiments with purified proteins demonstrate that the inhibitory function of NifL is activated by the non-uridylylated form of PII, but not by PII-UMP. Thus, unlike the *K. pneumoniae* NifL-NifA system where GlnK apparently has a positive role in relieving inhibition under N-limiting conditions, the inhibitory function of *A vinelandii* NifL is activated under N-excess conditions through interaction with PII-like regulatory proteins.

SYMBIOSIS-SPECIFIC TRANSCRIPTIONAL CONTROL OF *RHIZOBIUM ETLI RPON2*

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Recently, a second copy of *rpoN* was identified in *Rhizobium etli* CNPAF512, the symbiotic partner of *Phaseolus vulgaris* (common bean) (Michiels et al., 1998). *rpoN2* forms an operon with *orf180*, which is homologous to *y4vD* of *Rhizobium* sp. NGR234 (Freiberg et al., 1997). The transcription of *orf180-rpoN2* is solely dependent on RpoNl and NifA, under free-living microaerobic conditions. However, in bacteroids, there is still high expression of *orf180-rpoN2* in an *rpoN1* mutant background, which indicates the presence of an alternative, symbiosis-specific transcriptional control (Michiels et al., 1998). This symbiosis-specific transcriptional control is currently under investigation.

The region flanking the *orf180-rpoN2* operon was sequenced in search for genes that are involved in the symbiosis-specific expression of the operon. The sequenced region displayed an extensive resemblance with a gene region on the symbiotic plasmid of *R.* sp. NGR234 (Freiberg et al., 1997). Directly downstream of *orf180-rpoN2,* an operon containing at least three *orf*'s was found: *orfl05-nifU-nifS. orf105* is homologous to y4vC of *R.* sp. NGR234, and encodes a 105 aa putative protein belonging to the hypothetical *hesB/yadR/yfhF* protein family (Prosite PS01152). *R. etli nifU* encodes an hypothetical 316 aa protein, which is homologous to NifU of *Klebsiella pneumoniae,* but only for the first 100 aa. This is the first *nifU-like orf to* be identified in rhizobia. The 5' end of *nifS* was sequenced and is highly homologous to *nifS* of *Azotobacter chroococcum.* Further downstream of *orfl05* and *y4vC*, *fixABCX-nifA* is found (Michiels et al., 1998; Freiberg et al., 1997). Upstream of *orf180-rpoN2,* in the opposite orientation, *orf>74* is found, homologous to *y4vG of R.* sp. NGR234, which encodes a putative cytochrome P450 (Freiberg et al., 1997). *Orf180-rpoN2, orf105-nifU-nifS* and *orf> 74* all possess a putative NifA binding site and a –24/-12 consensus sequence, indicating that they probably all belong to the RpoN/NifA regulon.

There is an absolute necessity of RpoN/NifA for the expression of most of the *nif/fix* genes, hence for nitrogen fixation (Fischer, 1994). Bean plants inoculated with different *R. etli* mutants were grown at different temperatures. Temperature had no significant effect on the nitrogen fixation levels of plants inoculated with wild type *R. etli.* Bean plants inoculated with *nifA* mutants and non-inoculated plants showed no nitrogen fixation. At 26°C and to a lesser extent at 22°C, but not at 20 °C and 18 °C (Michiels et al., 1998), there was 10% of residual nitrogen fixation detectable with the *rpoN* double mutant. The **Fix$^+$** fenotype of the *rpoN* double mutant at 26 °C is a clear indication of the presence of a third *rpoN* copy, or of an alternative sigma-factor which can substitute for RpoN, in *R. etli.* This third copy appears to be under some kind of temperature-dependent control and could be responsible for the symbiosis-specific expression of *rpoN2.* However, despite extensive hybridisation and PCR experiments, we were not able to identify this third copy yet.

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GENETICS OF NITROGEN FIXATION IN *RHODOBACTER CAPSULATUS:* AMMONIUM AND MOLYBDENUM CONTROL OF BOTH NITROGENASE SYSTEMS

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The phototrophic purple bacterium *R. capsulatus* harbours two distinct nitrogenase systems: the conventional molybdenum nitrogenase (*nif* encoded) and an alternative, iron-only nitrogenase (*anf* encoded). As summarized in Fig. 1, the expression and activity of both nitrogenase systems is regulated by a number of environmental signals and occurs at least at four different levels.

1. Transcriptional control of *nifAI*, *nifAII*, and *anfA*

Genes encoding transcriptional activator proteins specific for the molybdenum nitrogenase (*nifAI, nifAII*) or specific for the alternative nitrogenase (*anfA*) are activated by NtrC-P under conditions of nitrogen depletion. Transcription of *anfA* is additionally negatively controlled by the molybdenum-dependent repressor proteins MopA and MopB. Thus, expression of *anfA* occurs only under nitrogen limiting conditions in the absence of molybdenum. In addition to NtrC, two further proteins are involved in signalling the nitrogen status of the cell: NtrB and GlnB. As found for many bacterial species, *R. capsulatus* harbours a second gene encoding a GlnB-like protein (*glnK*), which is cotranscribed with a gene encoding a (methyl)-ammonium transporter (*amtB*). It could be shown that expression of the *glnK-amtB* operon is regulated in an NtrC-dependent manner. In strains carrying lesions in *glnB* or in *glnB/glnK* double mutants, *nifAI, nifAII* and *anfA* are expressed even in the presence of ammonium. In contrast, *glnK* mutants are not affected in NtrB/NtrC-dependent ammonium control indicating that only GlnB is able to interact with NtrB.

2. Post-translational control of the activity of NifA and AnfA

Strains carrying constitutively expressed *nifA* or *anfA* genes were unable to express *nifH* or *anfH,* respectively, in the presence of ammonium indicating a post-translational mechanism controlling the activity of these activator proteins. This post-translational ammonium control of NifA activity was released in a *glnB/glnK* double mutant, whereas strains defective in either *glnB* or *glnK*, respectively, showed no effect on ammonium control. This result demonstrates that GlnB as well as GlnK are able to regulate directly or indirectly NifA activity. Interestingly, the post-translational ammonium control of AnfA activity was not affected in a *glnB/glnK* double mutant indicating that control of NifA and AnfA comprise two different mechanisms. In addition to post-translational ammonium control, the activity of AnfA is also affected by molybdenum. This molybdenum regulation was shown to be independent of the repressor proteins MopA and MopB.

3. Post-transcriptional control by mRNA processing

Intramolecular processing of mRNA was observed for the *nifHDK* and the *orf14-fdxC-fdxN-rnfF-orf10* transcripts *of R. capsulatus* (Schatt et al., 1989; Willison et al., 1993). To date, no further information about the mechanism of this post-transcriptional control is available and therefore, one can only speculate whether environmental signals are also involved in this process.

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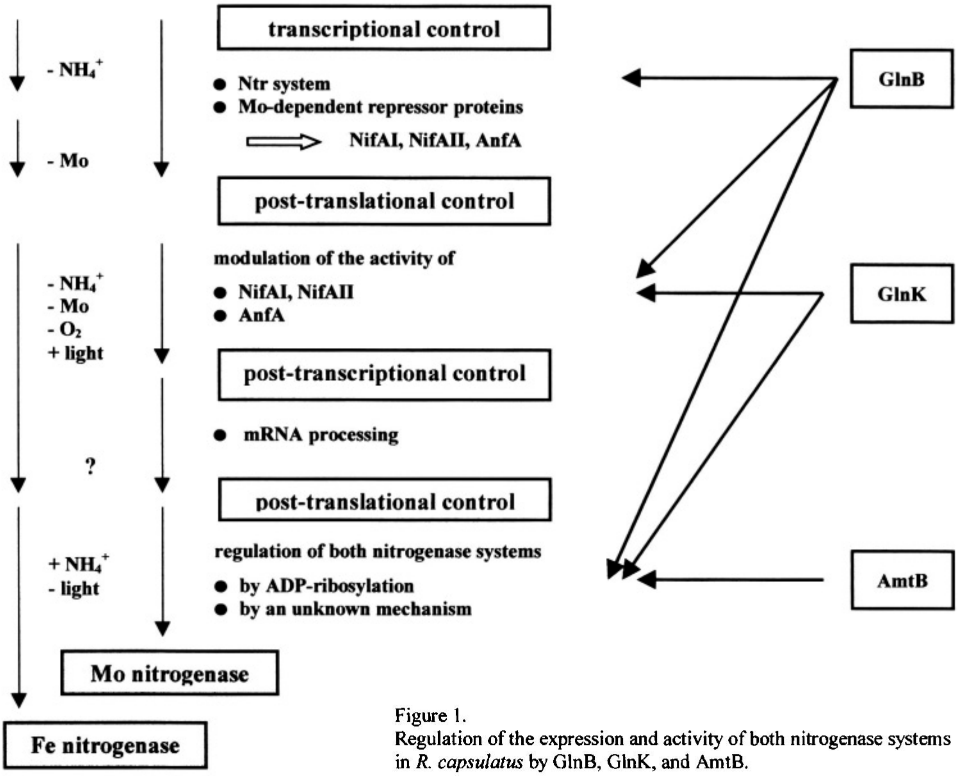
- NH_4^+
- Mo
transcriptional control
● Ntr system
● Mo-dependent repressor proteins
NifAI, NifAII, AnfA
post-translational control
modulation of the activity of
- NH_4^+
- Mo
- O_2
+ light
● NifAI, NifAII
● AnfA
post-transcriptional control
● mRNA processing
?
post-translational control
regulation of both nitrogenase systems
+ NH_4^+
- light
● by ADP-ribosylation
● by an unknown mechanism
Mo nitrogenase
Fe nitrogenase
GlnB
GlnK
AmtB

Figure 1.
Regulation of the expression and activity of both nitrogenase systems in *R. capsulatus* by GlnB, GlnK, and AmtB.

4. Post-translational control of nitrogenase activity

The activity of nitrogenase is controlled by reversible ADP-ribosylation of NifH or AnfH mediated by DraT and DraG. In addition, nitrogenase activity is regulated by a second mechanism not involving ADP-ribosylation of nitrogenase reductase (Pierrard et al., 1993). Analysis of a *glnB/glnK* double mutant demonstrated that not only transcriptional control of *nif* gene expression by ammonium but also both post-translational control mechanisms of nitrogenase activity are relieved. Even in the presence of ammonium, nitrogenase activities were found to be 4-fold higher in the *glnB/glnK* double mutant compared to the wild type under fully derepressing conditions. As described in detail by Yakunin and Hallenbeck (this volume), the (methyl)-ammonium transporter AmtB is also essential for ADP-ribosylation in response to ammonium.

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ELECTRON TRANSPORT PATHWAY TO NITROGENASE IN *RHODOBACTER CAPSULATUS*: RNF COMPLEX AND ITS RELATIVES IN NON-DIAZOTROPHS

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Supply of reducing power to nitrogenase is one of the limiting factors of nitrogen fixation, however, the electron pathway(s) to ferredoxin or flavodoxin which donates electrons to nitrogenase is poorly understood in most bacteria. Ferredoxin I encoded by the *fdxN* gene and flavodoxin encoded by the *nifF* gene are major electron donors to nitrogenase in *Rhodobacter capsulatus.* In the vicinity of *fdxN*, several genes have been identified to be essential for diazotrophic growth and designated as *rnf* (*rhodobacter nitrogen fixation*) genes (Saeki et al 1993; Schmehl et al 1993; Masepohl and Klipp 1996). Products of the *rnf* genes comprise trans-membrane proteins and proteins with Fe-S cluster(s). Some of them are detected as integral and peripheral proteins at chromatophore membranes and seem to stabilize each other to form a protein complex that is expected to be an energy-coupling FdxN reductase (Kumagai et al 1997; Jouanneau et al 1998). We report here 1) membrane topology of RnfD and RnfE proteins, 2) Rnf specific EPR signal, 3) co-purification of RnfC with $(His)_6$-tagged RnfB protein, and 4) identification of two genes downstream of *fdxN.*

1. Topology of membrane-spanning subunits of Rnf complex

Among *rnf* gene products, RnfA, RnfD and RnfE are highly hydrophobic proteins with similarity to the membrane-spanning subunits of Na^+-translocating NADH-quinone oxidoreductase in *Vibrio alginolyticus.* Analysis of 12 translational fusions of *rnfD* to *Escherichia coli* alkaline phosphatase *gene* (*phoA*) indicated that RnfD spans membrane at least 6 times with its central large hydrophilic regions exposed to periplasm while its N- and C- terminal regions reside in cytoplasm. PhoA fusions of RnfE protein indicated that RnfE spans membrane 6 times with N- and C- terminal regions reside in cytoplasm, a topology completely opposite to its close homologue RnfA.

2. Detection of EPR signal specific for Rnf Products.

Chromatophore membranes were prepared anaerobically from *R. capsulatus* cells grown under various conditions. Samples from diazotrophically grown and pseudo-diazotrophically grown wild-type cells showed an EPR signal at g=1.83, whereas samples from non-diazotrophically grown wild-type cells and from pseudo-diazotrophically grown *rnf*-null strains did not. However, it is still to be determined which of the putative FeS proteins, RnfB and RnfC, is responsible for the signal.

3. Co-purification of RnfC with RnfB-$(His)_6$ protein by Ni-NTA agarose chromatography.

RnfB protein with C-terminal addition of hexahistidine-tag is capable to support diazotrophic growth when introduced to non-polar *rnfB*-mutant strain. When membrane proteins were solubilized with dodecylmaltoside at pH 6.5, RnfC was co-adsorbed to Ni-NTA agarose, whereas no RnfC was adsorbed without $(His)_6$-tagged RnfB protein. These indicated that RnfB and RnfC associate together possibly as a subcomplex.

4. Identification of two genes downstream of *fdxN* : Dissection of rnfF two genes, *rnfX* and *rnfY*

Since there are some discrepancies between the two nucleotide sequences reported for the downstream region of *fdxN* (Saeki et al 1991; Schmehl et al 1993), we have re-determined the region and found two open reading frames in the *rnfF* locus identified by Schmehl et al (1993). To

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determine the number of genes in the region, we have made 8 insertion mutants and performed complementation analysis with DNA fragments containing downstream and upstream deletions; for upstream deletion, which lacked authentic promoter, the promoter for *pufQ* gene was used. The results of complementation experiments indicated that there are two independent genes (Table 1). Analysis of translational fusions of these genes to *E. coli LacZ* and *phoA* indicated that the product of the gene just downstream of *fdxN* situates at chromatophore membrane with its C-terminus exposed to cytoplasm. Here, we propose to call the genes, *rnfX* and *rnfY.*

Table 1. Genes of *rnf-fdxN* region and Their Products

Product	Residues	M_r	Properties
RnfA	193	20,424	Trans membrane protein; NqrE homologue [c]
RnfB	187	19,090	Peripheral membrane protein, 2 x [4Fe-4S] and [2Fe-2S] clusters ? [a-d]
RnfC	519	55,584	Peripheral membrane protein, 2 x [4Fe-4S] clusters ? [b, c]
RnfD	358	37,814	Transmembrane protein; NqrB homologue [b]
RnfG	217	22,846	NqrC homologue [d]
RnfE	237	26,277	Transmembrane protein; NqrD homologue [b, d]
RnfH	85	9,539	? [d]
FprA	435	47,894	FMN-protein
FdxC	95	10,163	Ferredoxin, [2Fe-2S] cluster
FdxN	64	6,733	Ferredoxin I, 2 x [4Fe-4S] clusters
RnfX	159	16,188	Membrane protein. Fe-S cluster ?; RseC, MucC homologue
RnfY	366	38,309	Secretion signal ?; NosX, ApbE homologue

[a]Saeki et al 1993, [b] Schmehl et al 1993, [c] Kumagai et al 1997, [d] Jouanneau et al 1997

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PHOTOSYNTHESIS IN AESCHYNOMENE *BRADYRHIZOBIUM SP.* ORS278: GENETIC ANALYSIS AND ROLE IN SYMBIOSIS

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1. Introduction

Bradyrhizobia isolated from particular stem-nodulated *Aeschynomene* species have the rare property among rhizobia to be photosynthetic. Such photosynthetic rhizobia specifically nodulate these *Aeschynomene* species and form a separate sub-branch on the *Bradyrhizobium* rRNA lineage distinct from *B. elkanii* and *B. japonicum* (Molouba et al., 1999).
The strain BTAi1, isolated from stem nodules of *A. indica* was the first rhizobium shown to ,develop a photosynthetic system (Eaglesham et al. 1990). Physiological studies, such as light induced CO2 or light depressed O2 uptakes, and photochemical measurements, gave evidence of the photosynthetic activity of BTAi1 (Evans et al. 1990; Hungria et al. 1993). This strain exhibits a photoheterotrophic and strictly aerobic photosynthesis. However, this organism was never studied at a genetic level and the role of the bacterial photosynthesis in the symbiotic interaction has not been truly investigated.
In photosynthetic bacteria, most of the genes required for the formation of the photosystem are clustered in a 45-kb region, designated the photosynthetic gene cluster (PGC). The PGC includes *bch* and *crt* genes coding enzymes for bacteriochlorophyll and carotenoid synthesis and the *puf* operon coding proteins of the light harvesting complex (*pufB* and *pufA*) and the proteins of the reaction center complex (*pufL and pufM*).

We report here the isolation and characterization of the *puf* operon from a photosynthetic *Bradyrhizobium* sp., strain ORS278, isolated from *Aeschynomene sensitiva.* We constructed a non-photosynthetic *PufLM-* mutant of strain ORS278. Preliminary results regarding the effects of such a mutant on the plant-bacteria symbiosis are presented.

2. Sequencing and genetic organization of the *puf* operon

A genomic bank of ORS278 was constructed in a colE1 vector to isolate photosynthetic genes using the following strategy: We designed degenerated primers by comparing PufL and PufM sequences from different photosynthetic bacteria (*Erythrobacter* sp. , *Rhodobacter capsulatus*, *R. sphaeroides* and *Rubrivivax gelatinosus)* and identifying well conserved domains. PCR amplification using degenerated primers defined from these domains gave fragments at the expected size whose sequence shared high similarities *with pufL* and *pufM* genes.
Next, two specific primers based on the sequence of the amplified DNA fragments were designed and used for PCR screening of the DNA genomic bank. Six positive clones containing insert from 35 to 45-kb were isolated and confirmed by Southern blot analysis. A common 4-kb region hybridising with a *puf* probe was sequenced. This region contains four ORFs, each of which had a Shine-Dalgarno sequence preceding the start codon, ATG. Comparisons with the *puf* genes of other photosynthetic bacteria revealed that these four ORFs were *pufB*, *pufA*, *pufL* and *pufM.* No other ORFs were found, indicating that the *puf* operon is only constituted by these four genes in strain ORS278.
Phylogenetic analysis of *pufLM* sequences of alpha-proteobacteria shows that the *puf* genes of strain ORS278 are closely related to those of the anaerobic photosynthetic bacteria *Rhodospeudomonas palustris* (supported by a bootstrap value of 99%) while they are phylogenetically distant from the aerobic photosynthetic bacteria *Roseobacter denitrificans* and

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Erythrobacter longus. A similar result was obtained with the 16S-rDNA phylogenetic analysis, indicating that the photosynthetic *Bradyrhizobium* strain ORS278 and *Rhodospeudomonas palustris* derive from a common photosynthetic ancestor.

3. Expression of *puf* genes in stem nodules

To investigate the role of the bacterial photosynthesis during symbiosis, we studied the expression of *puf* genes in endosymbiotic bradyrhizobia using *Escherichia coli lacZ* as reporter gene. A lacZ-kanamycin-resistant cassette replaced an 800bp fragment in the cloned pufLM genes. A chromosomic *pufLM-lacZ* fusion was constructed by recombinating the *puf-lacZ* construction to its chromosomal location using the suicide vector PJQ200.

Aeschynomene sensitiva plants were stem- and root-inoculated with this *pufLM-lacZ* mutant. No beta-galactosidase activity could be visualised in root nodules. By contrast a high beta-galactosidase activity was observed in all the stem nodule, indicating that the photosynthetic *puf* genes are expressed during stem symbiosis. This stem nodule specific expression of the photosynthetic genes was further confirmed by pigment analysis revealing the presence of Bchl only in stem nodules.

4. Symbiotic properties of a non-photosynthetic mutant

The *PufLM-* mutant was used to investigate the impact of photosynthetic genes on both root and stem nodulation and symbiotic nitrogen fixation.

No significant differences in plant growth and color foliage could be observed between *A. sensitiva* plants root-inoculated with wild type ORS278 or the *PufLM-* mutant over a two month-period. In contrast stem nodulation of *A. sensitiva* by the *Puf-* mutant was delayed (about 5 days) and the number of stem nodules was dramatically reduced (two- to threefold) when compared to that for the wild type strain. A high reduction in two month-old plant growth (about 50% reduction) and nitrogenase activity (60% reduction in ARA) were also observed.

5. Conclusion

Our results clearly show that the bacterial photosynthesis plays a key role during the stem symbiosis between *Bradyrhizobium* sp. ORS278 and its host plant *A. sensitiva.* This photosynthesis appears to be important for the infection process perhaps by facilitating the survival of the bacteria on the stem or by providing energy necessary for stem infection. Moreover this photosynthesis appears to be active in stem symbiosis, as indicated by expression of *puf-lacZ* fusion in stem nodules during the symbiosis. Photosynthesis may directly furnish energy to the bacteroid, thus limiting the demand for plant photosynthate and thus may promote nitrogen assimilation and plant growth.

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THE CONCERTED ACTION OF HYDROGENASES AND NITROGENASES IN CYANOBACTERIA

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In addition to the Mo-containing nitrogenase, cyanobacteria can express alternative enzymes. Under Mo-deficiency, but in the presence of V, a V-containing enzyme is synthesized in the filamentous, heterocystous cyanobacterium *Anabaena variabilis* (Kentemich et al. 1988). The genes coding for the V-nitrogenase have extensively been characterized (Thiel 1993). Also, there are some physiological indications that an Fe-only nitrogenase is expressed in the absence of both Mo and V in the medium (Kentemich et al. 1991). These two alternative nitrogenases produce more H_2 than the Mo-enzyme and reduce C_2H_2 to C_2H_4 and (~3%) C_2H_6; the latter is indicative for their expression in cyanobacteria, *Azotobacter* and other prokaryotes (Loveless, Bishop 1999). In Nature, conditions have not been found under which alternative nitrogenases are expressed. A recent screening project at the Baltic-Sea island of Hiddensee (Hübel, Hiddensee, Bothe, unpublished) gave no indication for the expression of alternative nitrogenases in the filamentous cyanobacteria occurring there (*Calothrix* spp., *Anabaena torulosa, Rivularia atra* and *Nodularia spumigena*). Independently, two groups (Schrautemeier et al. 1995; Thiel et al. 1995) made the remarkable observation that *A. variabilis* synthesizes a second, Mo-enzyme under reduced oxygen tensions. The genes encoding this enzyme have been sequenced. The enzyme is expressed in vegetative cells, which contrasts with the original Mo-nitrogenase that is restricted to heterocysts. Its properties are similar to the nitrogenase of the filamentous, non-heterocystous *Plectonema boryanum.* In cyanobacteria, alternative nitrogenases have been found in *A. variabilis* and in *A. azollae* of the water fern *Azolla*, but not in any other cyanobacterial species (Thiel 1993).

All cell-free nitrogenase preparations produce H_2 simultaneously with N_2 reduction. In intact cells, very little net H_2 is evolved due to recycling by hydrogenases (Houchins 1984; Papen et al. 1986). This H_2-uptake benefits organisms. First, H_2 is consumed in a respiratory-dependent oxyhydrogen (Knallgas) reaction, removing it from the nitrogenase site. In addition, this reaction might provide ATP which minimizes the loss of energy during H_2-formation by nitrogenase. Indeed, intact *A. variabilis* cells are significantly more resistant to O_2 when assays are supplemented with H_2 (Papen et al. 1986). Further, H_2-uptake by hydrogenases may provide additional reductant for nitrogenase and may protect against H_2 build-up, which can inhibit nitrogenase reactions at high concentrations.

Cyanobacteria possess at least two hydrogenases (Houchins 1984). The uptake hydrogenase catalyzes *in vitro* the consumption of H_2 in the presence of phenazine methosulfate or methylene blue but not either dithionite- or methyl viologen-dependent H_2-production. In *Anabaena* PCC7120, the gene encoding the larger subunit (*hupL)* rearranges prior to expression and in parallel with heterocyst differentiation (Matveyev et al. 1994; Carrasco et al. 1995). It is still unclear whether the uptake hydrogenase is confined to heterocysts or is also present in vegetative cells (Peterson, Wolk 1978; Lockau et al. 1978; Eisbrenner et al. 1981). A mutant of the unicellular, non-N_2-fixing *Anacystis nidulans* could perform PMS–dependent H_2-uptake to about 50% of maximal activity, but was completely inactive in H_2-evolution (Boison et al. 1996), clearly indicating an uptake hydrogenase in non-N_2-fixing cells. Newer data (Boison, Bothe, Schmitz, unpublished) show that part of *hupL* (coding for the larger subunit of the uptake hydrogenase) can be amplified by RT-PCR, using suitable primers from the *hupL* sequences and a DNA-free mRNA preparation from ammonia-grown *A. variabilis.* Since ammonia-grown cells contained absolutely no heterocysts, the uptake hydrogenase is expressed at least at low level also in non-N_2-fixing, heterocyst-free filaments of *Anabaena variabilis.* The other hydrogenase is the bidirectional enzyme. Immunogold-

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labelling indicated its presence at the cytoplasmic membrane in *Anacystis nidulans* (Kentemich et al. 1990). The genes encoding this enzyme were sequenced from *A. variabilis* and are related to the $NAD(P)^+$-dependent hydrogenases from *Alcaligenes eutrophus* and *Desulfovibrio fructosovorans* (Schmitz et al. 1995; Boison et al. 1998). The genes are *hoxFUYH,* which are interspersed in *A. variabilis* with two ORFs. Extracts from *Anacystis nidulans* catalyze an H_2-dependent reduction of $NAD(P)^+$ and NAD(P)H-dependent H_2-formation (Schmitz, Bothe 1996). The gene arrangement is remarkably dissimilar in three cyanobacteria so far tested. In *Synechocystis* PCC 6803, ORFs, different from *Anabaena variabilis* in DNA sequence and position, are located between the *hox* genes, and an additional hydrogenase gene *hoxE* resides upstream of *hoxF* (Kaneko et al. 1996; Nakamura et al. 1998; Appel, Schulz 1996). In *Anacystis nidulans*, *hoxUYH* are contiguous, and the genes *hoxW*, *hypA*, *hypB* and *hypF* coding for accessory genes reside downstream of *hoxH.* The genes *hoxEF* are separated by 16 kb from this cluster in *A. nidulans* (Boison et al. 1998, 1999). New transcriptional analyses by RT-PCR (G. Boison, H. Bothe, O. Schmitz, unpublished) show that *hoxUYHWhypAB* form a transcriptional unit, whereas *hypF* may be transcribed separately. The genes *hoxEF* form another transcriptional unit. In *A variabilis*, the *hoxFUYH* cluster constitutes a transcriptional unit including the two ORFs.

Future work will identify promoters to study hydrogenase-gene expression. Primer-extension and promoter expression studies are underway. Defined mutants should reveal functions of individual subunits, particularly those of the ORFs. Further, double mutants (both in uptake and bidirectional hydrogenase) may have potential applications, possibly in solar-energy-conversion projects. Since some cyanobacteria thrive on inorganic salts only, can meet their N-demand by N_2-fixation, and have both hydrogenases most likely acting as H_2-consuming enzymes in intact cells of autotrophic species, a double mutant impaired in both hydrogenases could reveal the maximal capability for H_2-production catalyzed by nitrogenase. Such a construct would show whether cyanobacteria offer some alternatives to established solar energy conversion systems like photovoltaics.

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HETEROCYST PATTERN CONTROLLED BY AN INHIBITORY PEPTIDE IN *ANABAENA*

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The filamentous cyanobacterium *Anabaena* PCC 7120 produces a developmental pattern of single nitrogen-fixing heterocysts separated by approximately ten vegetative cells. We have identified a 54-bp gene, *patS*, that blocked heterocyst differentiation when overexpressed from a conjugal plasmid. A deletion strain (Δ*patS*) produced supernumerary heterocysts and abnormal spacing. Synthetic peptides corresponding to the COOH-terminal five or six residues blocked heterocyst development when added exogenously to wild-type filaments. Peptide also inhibited heterocyst formation in the Δ*patS* mutant, but failed to restore a normal pattern, showing that a uniformly supplied signal is not sufficient for producing normal heterocyst spacing. A wild-type phenotype was produced when the Δ*patS* mutant was complemented by *patS* driven by an early heterocyst-specific promoter (*hepA*), showing that PatS produced in proheterocysts inhibited nearby cells. A *patS*-*GFP* fusion was used to follow temporal and spatial regulation of *patS* expression. After 12 hours, GFP fluorescence was detected exclusively from proheterocysts. These data indicate that a PatS signal, probably a processed COOH-terminal peptide, is produced by differentiating cells and inhibits nearby cells by diffusion, probably through the periplasmic space. Epistasis experiments indicate that *patS* functions early in heterocyst development.

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THE ELEMENT NITROGEN IN THE PHILATELIC WORLD

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This special poster is on display for the first time and it reflects my special love for both dinitrogen fixation and stamp collecting. Many aspects of the nitrogen cycle can nicely be demonstrated by stamps. The history related to the element nitrogen can be traced back even to the time of the Greeks time and ends (on stamps) with A. Virtanen.

Major contributors to the subject can be displayed, however, I did not find stamps celebrating either Boussingault or Hellriegel. Only a few examples can be shown from the ~13,000 sppecies of legumes, most of which form a symbiosis with *Rhizobium.* There are many opportunities to demonstrate species from the Mimosaceae, Caesalpiniaceae and Fabaceae. With regard to the *Frankia* symbiosis, good examples exist on stamps for *Hippophae rhamnoides* (sea buckthorn), *Casuarina*, *Myrica* and also *Alnus.* With cyanobacterial symbiosis, I could not find an example with *Azolla,* but a nice series of Chinese issues with different cycads exists. The fascinating carnivores (meeting their N demand partially by catching animals) are exemplified by stamps with sundew (*Drosera rotundifolia*), *Aldrovanda*, pitcher plant (*Sarracenia purpurea*), butterwort *(Pinguicula)* and *Nepenthes* but, most surprisingly, I did not find a single example of the Venus fly trap (*Dionaea*).

The element N is connected with aspects like pollution by either acid rain or NO_x-formation, dynamite and its stabilisation (Nobel), and solar-energy conversion to molecular H_2 by N_2-fixing microorganisms. Many natural compounds, particularly secondary metabolites that contain N, like alkaloids and pseudoalkaloids, and plants forming such substances are often beautiful and, therefore, frequently used on stamps.

I enjoyed myself setting up this somewhat laborious poster and cordially invite you to join the club of the stamp-collecting N_2-fixation community.

Acknowledgement

This work was significantly supported by the patience of our photographer, Siegfried Werth, and my wife, Gundel.

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SECTION III

TAXONOMY AND EVOLUTION OF DIAZOTROPHS

TAXONOMY AND EVOLUTION OF DIAZOTROPHS

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The past decade has seen a revolution in our understanding of bacterial phylogeny and an accompanying resurgence in taxonomic activity. Inspired by the establishment of small subunit (16S) ribosomal RNA as a universal marker, powered by advances in DNA sequencing technology, and encouraged by more accessible phylogenetic software, a new breed of DNA-oriented taxonomist has emerged. New species have proliferated, and it would be almost unthinkable now to publish a new species without a 16S sequence to establish, apparently beyond doubt, its precise place in the bacterial firmament. At the same time, our wider knowledge of bacterial genomes has increased immensely. Whole genomes have been sequenced, including the first diazotroph genome, that of *Methanobacterium thermoautotrophicum.* As we learn more about genomes, we need to reassess the criteria by which we partition the bacterial world into genera and species, and to define our rationale and objectives more clearly.

Do bacterial species correspond to natural biological units, or are they merely human attempts to partition a continuum? Mechanisms that could in principle maintain coherent species are of two types. Genetic cohesion mechanisms involve sufficient gene exchange between members of a species to maintain a common gene pool, but greatly reduced exchange between species. Ecological cohesion mechanisms can maintain the discreteness of even a clonal species provided that its members share a common niche. Studies of diversity have shown that at least some bacteria, e.g. enterics, do have cohesive species, but there is no certainty that this is universal.

One important relevant realization is that bacterial genomes are compound. In a simplified conception, we can think of a bacterial genome as consisting of two parts. The basic genome is composed of housekeeping genes that are needed under almost all growth conditions, they are carried on the chromosome (or chromosomes), their organization is reasonably stable, and they are predominantly inherited vertically from mother to daughter cells. By contrast, the accessory genome consists of genes that are entirely selfish or offer adaptations to special circumstances, these are carried on plasmids, islands, transposons and phages, they undergo frequent rearrangement and are often transferred horizontally between cells. Of course the distinction between basic and accessory genomes is not absolute, but as we study genome structures more extensively we should find out whether it is close enough to reality to provide a useful framework. Because of the varied transfer agents involved in the accessory genome, the extent of gene exchange in the accessory genome is likely to be specific to the individual components, so it is likely that this genome will not define clear and consistent species boundaries. If species exist, they need to be defined with reference to the basic genome, which includes 16S genes of course. It is ironic, though, that genetic cohesion depends on gene exchange and ecological cohesion depends on niche adaptation, and both these aspects are largely determined by the accessory genome.

There have been some significant recent advances in our knowledge of the genome structure of diazotrophs. We are increasingly discovering that bacteria may have more than one chromosome; for example, Martin-Didonet et al. report in this volume that 16S genes may be on more than one replicon in *Azospirillum.* In rhizobia three sizes of replicon are generally distinguished: plasmids (<1MB), megaplasmids (1-2MB), and chromosome (>4MB, so far as known). The organization differs: *Rhizobium leguminosarum* has a chromosome and 2-8 plasmids, *Sinorhizobium meliloti* has

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a chromosome, two megaplasmids, and sometimes 1-2 plasmids, *Mesorhizobium* has a chromosome, but may lack other independent replicons. In each case the nodulation genes are carried in the accessory genome: on a plasmid, a megaplasmid, and a symbiotic island inserted in the chromosome, respectively. Intriguingly, the *S. meliloti* genome sequencing project has revealed (Hernandez-Lucas et al. this volume) that the pExo megaplasmid has the replication and partition operon *repABC* and a putative origin of replication closely similar to those commonly found in smaller plasmids of rhizobia and agrobacteria (Turner and Young, 1995; Rigottier-Gois et al., 1998). It seems that a megaplasmid is just an ordinary plasmid grown large, and it will be fascinating to discover, as the genome is completed, whether this is also true of the other megaplasmid and even, perhaps, of the chromosome itself.

These insights into genome organization have important implications for the practice of bacterial taxonomy. An essential tool for the delineation of new species is the determination of DNA-DNA reassociation. High levels of heteroduplex formation between DNA from two isolates is evidence that they belong to the same species, while a level that is below a threshold is taken to indicate that they are different species. In order to describe a new species, it is necessary to show that the isolates are sufficiently diverged from representatives of other species by this criterion. This is enshrined in the published guidelines, e.g. Graham et al 1991. Unfortunately, there is more than one methodology for measuring heteroduplex formation, the precise criteria differ between laboratories, and the methodology is sensitive to small variations that make it hard to replicate. Furthermore, it is time-consuming and, because it involves pairwise comparisons, it is impractical to test as many strain combinations as would really be desirable. Despite all these drawbacks, some very good studies of DNA-DNA hybridization have been made, and have resulted in stable, well supported species definitions. The major problem that is highlighted by current progress in genome research is that we really do not know exactly what DNA-DNA hybridization is measuring. When it first came into use, the general picture was that different bacteria would carry essentially the same complement of genes, but that each gene would be diverged at certain percentage of nucleotides depending on the relatedness of the bacteria. However, we now know that a major fraction, perhaps even half, of the DNA may belong to the accessory genome, that large suites of genes may be gained or lost in isolates that remain chromosomally identical, and that distantly related bacteria may, through lateral transfer, share elements that are identical. If we are to continue to take DNA hybridization seriously, we urgently need to investigate the effects of these phenomena on the results.

The papers in this section reflect many of the themes I have explored. Martinez-Romero et al. discuss the relationship between rhizobia and *Brucella*, the intertwining of their phylogenies is the result of lateral transfer of accessory genes, especially the symbiosis genes. On a larger phylogenetic scale, Young considers the evolution of nitrogen fixation, a sporadically-distributed phenotype that has led to spurious taxonomic groupings in the past. Van Berkum presents sequence data from 23S rRNA and RnaseP, both “housekeeping” genes, in support of the 16S phylogeny of rhizobia. Cyanobacteria have long been a taxonomic wasteland, but Rasmussen shows that molecular tools provide a firm way forward. Many other contributions in this volume also demonstrate the power of molecular methods to give unambiguous answers to important taxonomic and evolutionary questions.

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ECOLOGICAL, PHYLOGENETIC AND TAXONOMIC REMARKS ON DIAZOTROPHS AND RELATED GENERA

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1. Introduction

Nitrogen-fixing organisms are very diverse prokaryotes that have been identified scattered in the phylogenetic trees reconstructed from the comparative analysis of ribosomal RNA gene sequences (Martinez-Romero, 1985, Young, 1992). Although 16S rRNA gene-based phytogenies have been criticized and the Universal Tree of Life questioned (Pennisi, 1998), novel approaches from proteome analysis derived from genomes of 20 organisms provide support to the unrooted Universal Tree of Life based on 16S rRNA genes (Tekaia et al., 1999). However, the final answer has not yet been given in this highly controversial field. A critical overview of 16S rRNA gene based phylogenies compared to other markers has been presented (Martinez-Romero et al., 1999). Anyhow, it is clear that nitrogen fixers are found in close relationship to non nitrogen fixers. Nitrogen fixers may constitute clusters restricted in distribution inside groups of non-fixing bacteria as is the case of *Acetobacter diazotrophicus* and novel *Acetobacter* N-fixing species inside *Acetobacter* species (Caballero-Mellado et al., 1999; Fuentes-Ramírez et al, this volume) or *Paenibacillus* among aerobic endospore-forming Firmicutes (Achouak et al., 1999). In Archea, N-fixation is a general property of the methanogen group and *nif* genes are found in halophiles, but no fixers have been found in the sulfur dependent archaeobacteria. The *Frankia* group in the actynomycetes includes atypical strains as well as an infective but no nitrogen fixer isolate (Normand et al., 1996); in *Rhizobium* there are no nitrogen fixers, nonsymbiotic strains (Segovia et al., 1991), meaning that nitrogen fixation may be easily lost in some cases. The loss of nitrogen fixing ability has been proposed to explain non fixers occurring closely related to N-fixing bacteria.

Possessing nitrogen fixing genes would not be advantageous for bacteria if they inhabit N-rich environments. N-fixers are beneficial in plant-bacteria associations, unfortunately, farmers provide N-rich media when heavily fertilizing agricultural fields and we supposed that these practices may negatively affect populations of nitrogen fixers (Martinez-Romero, Caballero-Mellado, 1996). We have documented evidence of different negative effects of fertilization: lack of recovery (Fuentes-Ramírez et al., 1993) and less genetic diversity of *A. diazotrophicus* in fertilized sugar cane fields (Caballero-Mellado et al., 1995) with diminished colonization by *A. diazotrophicus* of N-fertilized sugar-cane plants (Fuentes-Ramírez et al., 1999), Fig. 1. We also reported a decrease in the genetic diversity of *Rhizobium* recovered from *P. vulgaris* (bean) nodules in fertilized fields using the commonly recommended N dose for bean fertilization in Mexico (Caballero-Mellado, Martinez-Romero, 1999), Fig. 2. It seems highly probable that not all nitrogen fixers are known at present. Some may be unculturable. "New findings may fill in some of the blanks on the bacterial map, and the distribution on N2 fixation may prove to be less patchy than it appears at present" (Young, 1992). However, very few real new N-fixers have been reported in these last years, this may be in relation to the fact that the ability to fix nitrogen is not a routine test for new isolates and is performed only in labs specializing in the subject. In addition, pathogenic bacteria are usually excluded from N-fixing analyses as they are supposedly considered non-fixers. Nevertheless,

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Agrobacterium tumefaciens strains, a plant tumor inducer, has *nif* genes and fixes nitrogen (Kanvinde, Sastry, 1990). Around 30% of klebsiellae, either soil or hospital isolates, are diazotrophic (Postgate, Eady, 1988) or even 100% of them may fix depending on assayed conditions (unpublished results). Furthermore, nitrogen fixation may be expressed only under special bacterial growth conditions. Many free-living organisms require anaerobic conditions for fixation. *Bradyrhizobium* requires a burst of glutamate; *Rhizobium* a differentiation process; *Azoarcus*, diazosome formation (Karg, Reinhold-Hurek, 1996) and in *Azospirillum* nitrogen fixation is stimulated by plant agglutinins (Karpati et al., 1999).

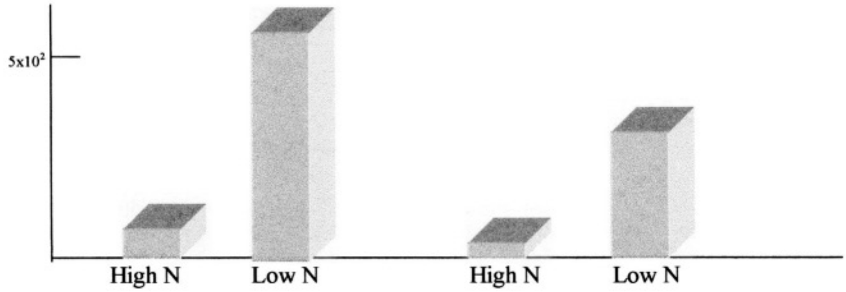

Figure 1. *A. diazotrophicus* colony forming units (CFU) recovered from nitrogen fertilized or non fertilized sugarcane plants at 30 days after sprouting.

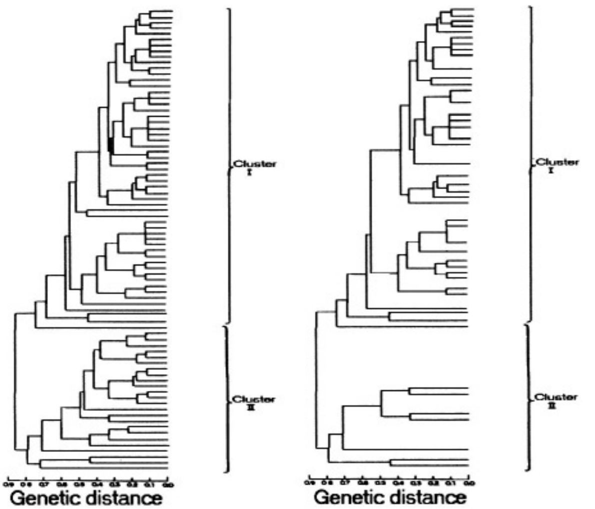
Cluster I
Cluster II
0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
Genetic distance
Cluster I
Cluster II
0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
Genetic distance

Figure 2. Electrophoretic types recovered from *Phaseolus vulgaris* bean nodules in non-fertilized (left dendrogram) and fertilized fields (N:P:K=40:40:0) (right dendrogram).

In any case, bioprospection for new fixers should be encouraged and this effort seems highly justified. We are searching for diazotrophs associated to banana plants.

The identification of new isolates and the phylogenetical classification that looks toward clustering natural groups are the basis for good science and are clearly related to important changes in family names of N fixers that would be adopted in the new Bergey's Manual. Rhizobiaceae will no longer encompass *Rhizobium, Bradyrhizobium* and *Mesorhizobium* species. *Mesorhizobium* will be included in a new family together with *Phyllobacterium* and *Bradyrhizobium* grouped with *Rhodopseudomonas* in another new family. *Azoarcus* will be distributed in several families.

2. Rhizobia and related genera

From the comparative analysis of 16S rRNA genes, relationships of bacteria to known fixers have been revealed. For example, strains of sewage water *Zooglea ramigera* were found to be similar to the rhizobia group (Rosselló-Mora et al., 1993); *Pseudoaminobacter, Chelatobacter,* and *Aminobacter* with important uses in biotechnology for bioremediation are groups in close relationship to *Mesorhizobium* spp (Auling et al., 1993; Kampfer et al., 1999); and the bacteria-lysing *Ensifer* isolates are similar to (*Sind*)*rhizobium meliloti* (D. Balkwill, personal communication). We have found that a new cluster of *Mesorhizobium* represented by SH0172 (Tan et al., 1999) share with methylotrophic bacteria the capacity to use methanol as sole carbon source.

Brucella constitute a cluster of bacteria in relationship to *Rhizobium* spp (de Ley, 1987; Martinez-Romero et al., 1991; Young et al., 1991). From 16S rRNA gene sequences the position of the nodes relating this genus to other rhizobia may not be confidently resolved (Ludwig et al., 1998). By multilocus enzyme electrophoresis we confirmed the close relationship of *Brucella* and *Rhizobium* using more than 100 *Brucella* isolates and *Rhizobium* reference strains (Fig. 3) and found that *Brucella* was closely related to *R. tropici.*

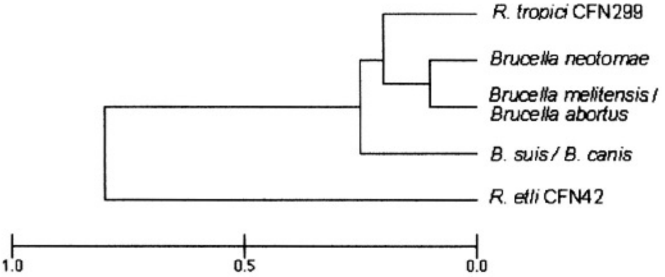

Figure 3. Genetic relatedness of *Brucella* and *Rhizobium* species deduced from the electrophoretic mobility of metabolic enzymes. Each line represents an electrophoretic type and may represent one or more strains.

All the *B. melitensis* and *B. abortus* strains studied have two chromosomes of sizes 2.1 and 1.15 Mb (Michaux et al., 1993). *B. suis* biovars 2 and 4 have two chromosomes with different sizes and a unique chromosome was found in *B. suis* biovar 3. An ancestor with a single chromosome has been proposed for all *Brucella* strains. Rearrangement at the *rrn* loci led to the existence of two minichromosomes (Jumas-Bilak et al., 1998). Our data clearly support a monochromosomic ancestor of *Brucella* that must have been related to *R. tropici* as well. It is interesting to remark that *R. tropici* is also closely related to *Agrobacterium* biovar 2 strains and *Agrobacterium* species have been recovered as clinical isolates in humans (Alnor et al., 1994; Riley, Weaver, 1977). *Brucella* chromosomes are in the range of size of the megaplasmids of *Rhizobium* and megaplasmids have been observed in *R. tropici* but these megaplasmids do not harbor rRNA genes (Geniaux et al., 1995). In addition, *R. tropici* has smaller plasmids one of them (≈ 500 Kb) harboring *nod* and *nif* genes (Martinez et al., 1987). We are pursuing the analysis of the genetic differences of *R. tropici* and *Brucella* towards the elucidation of mechanisms involved in the evolution of a pathogen, *bac* genes involved in bacteroid differentiation in *R. meliloti* (Glazebrook et al., 1996) have homologs in *Brucella* required for macrophage invasion thus providing further support to the close relatedness of both genera.

Table 1. Current taxonomy of rhizobia

Genus	Species	Host
Allorhizobium	*A. undicola*	*Neptunia natans*
Azorhizobium	*A. caulinodans*	*Sesbania rostrata*
Bradyrhizobium	*B. elkanii*	*Glycine max*
	B. japonicum	*Glycine max*
	B. liaoningense	*Glycine max*
Mesorhizobium	*M. amorphae*	*Amorpha fructicosa*
	M. ciceri	*Cicer arietinum*
	M. huakuii	*Astragalus*
	M. loti	*Lotus*
	M. mediterraneum	*Cicer arietinum*
	M. plurifarium	*Acacia, Leucaena*
	M. tianshanense	*Glycyrrhiza, Sophora, Glycine* and others
Rhizobium	*R. etli*	*Phaseolus vulgaris, Mimosa affinis*
	R. galegae	*Galega*
	R. gallicum	*Phaseolus vulgaris*
	R. giardinii	*Phaseolus vulgaris*
	R. hainanense	*Stylosanthes, Centrosema, Desmodium, Tephrosia*
	R. huautlense	*Sesbania herbacea*
	R. leguminosarum	*Vicia, Trifolium*
	R. mongolense	*Medicago ruthenica, Phaseolus vulgaris*
	R. tropici	*Phaseolus vulgaris, Leucaena*
Sinorhizobium	*S. arboris*	*Acacia senegal, Prosopis chilensis*
	S. fredii	*Glycine max*
	S. kostiense	*Acacia senegal, Prosopis chilensis*
	S. medicae	*Medicago* spp
	S. meliloti	*Medicago sativa*
	S. saheli	*Sesbania*
	S. terangae	*Sesbania, Acacia*
	S. xinjiangense	*Glycine max*

Bioprospection in *Rhizobium* has yielded fruit. The excellent work in NGR234 led by Broughton (Freiberg et al., 1997; Perret et al., 1999; Pueppke, Broughton, 1999; Viprey et al., 1998) has been possible after the pioneer work of bioprospection of legume symbionts by Trinick in New Guinea in 1965 (Trinick et al., 1980). Similarly, the *Rhizobium* species described in recent years, such as *Azorhizobium caulinodans*, *Rhizobium galegae*, *R. fredii, R. etli*, and *R. tropici*, have enriched our knowledge in this field. As an example, novel modifications of Nod factors have been recognized in new species (Bec-Ferté et al., 1994; Poupot et al., 1995; Mergaert et al., 1993) and in isolates as BR816 (Snoeck et al., this volume). BR816 is a *Sinorhizobium* isolate from the analysis of 16S rRNA sequences (Hernández-Lucas et al., 1995). Naming *R. tropici* as a new species (Martinez-Romero et al., 1991), encouraged research in different aspects including studies that led to the identification of sulfate on Nod factors (Poupot et al., 1993) that was otherwise exclusively encountered in *R. meliloti* (Roche et al., 1991). *R. tropici* strains have been extensively used as bean (*Phaseolus vulgaris*) inoculants in South America (Vlassak et al., 1997).

The identification of *M. amorpha* (Wang et al., 1999a) showed the clear existence of symbiotic plasmids containing *nif* and *nod* genes in *Mesorhizobium. M. loti* and *M. plurifarium* have these genes in the chromosome and in *M. loti,* symbiotic islands have been described (Sullivan, Ronson, 1998). This finding adds support *to Mesorhizobium* being intermediate between *Rhizobium* (having mainly symbiotic plasmids) and *Bradyrhizobium* (with symbiotic determinants in chromosome). *R. huautleme* (Wang et al., 1998) is the sole *Rhizobium* species related to *R. galegae,* that was otherwise mainly immersed in an *Agrobacterium* cluster (Young, Haukka, 1996). The *R. huautlense-Sesbania herbacea* symbiosis is highly adapted to flooded conditions (Wang, Martinez-Romero, submitted) without stem nodules. *R. etli* represents the main symbiont of *Phaseolus* in its site of origin (Segovia et al., 1993) . The proposal of a new biovar (mimosae) in *R. etli* isolates from *Mimosa affinis* (Wang et al., 1999b) allows the identification of a possible ancestral plasmid of *phaseoli* and presents clear data on fast plasmidic evolution. It also shows that biovars are a common feature in *R. etli, R. leguminosarum,* and *R. gallicum.*

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MOLECULAR EVOLUTION IN DIAZOTROPHS: DO THE GENES AGREE?

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1. Introduction

Nitrogen fixation occurs in a wide range of Bacteria and Archaea. Nevertheless, it has been reported in only around a hundred genera, covering a small fraction of known species. Thanks to the sequencing of complete genomes, we now know that a number of bacteria have nothing resembling the conventional nitrogenase genes, so the lack of detectable nitrogen fixation does not merely reflect our inability to provide appropriate conditions for expression. I would argue that this patchy distribution reflects a history of gene duplication and loss rather than multiple origins or promiscuous transfer. Phylogenies for the nitrogenase enzyme are presented and interpreted.

2. Why Character Distributions May Not Match Organismal Phylogeny

When a character, such as nitrogen fixation, is present in some groups of organisms but absent from other related species, there are three general classes of explanation. Firstly, the character may have had multiple independent origins. If so, then, underneath the superficial similarity, the detailed implementation of the character is likely to be different in each case. The recently discovered superoxide-based diazotrophic actinomycete provides such an example. This organism fixes N_2 using an enzyme system that is totally different from the nitrogenases used by all other known diazotrophs (Ribbe et al. 1997; O. Meyer et al, this volume). It may be an “independent invention”. Secondly, it could be that the character was present in the common ancestor of all organisms that now possess it, but has been lost many times in the lineages that no longer have it. In general, it is much more likely that a complex character will be lost than that it will arise. Thirdly, the character may only have arisen once, but then spread to other lineages by lateral gene transfer. Clearly, suitable gene transfer processes must exist: it is easier to imagine in bacteria than in mammals, say.

In general, we would expect the phylogeny for any gene to reflect the phylogeny of the organisms that carry it. However, this is sometimes not the case, and again there are three classes of explanation that mirror those discussed in the previous paragraph. Firstly, it is possible that the unexpected similarity of certain sequences is not a result of their recent common ancestry but reflects convergent evolution in response to a common selective pressure. Usually, it is possible to identify such cases because the rate of functionally important changes is enhanced relative to neutral substitutions. Secondly, some of the genes may be paralogous rather than orthologous: a gene duplication in an ancestor may give rise to two divergent gene lineages, with one or the other being deleted (or merely unobserved) in different organisms. In this case, if orthologous sets can be identified, the phylogeny of each should reflect that of the organisms. Thirdly, gene transfer and replacement may account for unexpected similarities in the genes.

For taxonomists, the comparative phylogeny of different genes is an important new source of information. Bacterial classification has in recent years become highly dependent on analysis of the small subunit (16S) ribosomal RNA genes. The major groups defined in this way (e.g. the proteobacteria and their subdivisions) have in general been confirmed by other characteristics, including the phylogeny of other genes, and provide a framework for considering the evolution of the nitrogenase genes (Young 1992, 1993).

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3. The Phytogeny of the Nitrogenase Genes

The phylogeny of the NifH proteins (dinitrogenase reductase or iron protein) is complex (Figure 1). It can be resolved into four classes A, B, C, D (Young 1990), and the many new sequences added since my last review (Young 1993) now give a fuller picture. Class D has the most divergent sequences, and it is unclear whether these proteins are actually part of nitrogenase systems. Indeed, a search of the complete *Methanococcus jannaschii* genome (Bult et al. 1996) does not reveal either *nifD*- or *nif*K-like genes. The other three classes are separate clades that apparently split almost simultaneously. There are new sequences in the A (alternative nitrogenases) and C (mostly clostridial) classes, but they leave these clades consistent with organismal phylogeny. Class B has been studied most widely and is represented by the largest number of sequences. Class B NifH has been found in the alpha, beta and gamma proteobacteria, in the cyanobacteria and in *Frankia* (in the high G+C gram positive group). On the whole, the phylogeny of NifH reflects these organism groupings. However, the alphas, the gammas, the cyanobacteria and *Frankia* are equally divergent from each other: the two proteobacterial groups are not closer, as one would expect from their ribosomal RNA relatedness. The beta proteobacteria cluster closely with the alphas in 16S rRNA analyses, so the position of the *Alcaligenes faecalis* NifH sequence (ALCFA) is not too surprising, but there is an unexpected grouping of *Thiobacillus ferrooxidans* (THIFE, beta) with three *Bradyrhizobium* and *Azorhizobium* sequences (BRAJA, BRASP, RHISO, alpha) that is not close to the expected position for any of these genera (being significantly more distant from the fast-growing rhizobia than *Rhodobacter, Rhodospirillum,* and *Azospirillum,* in contrast to the 16S tree). Egener et al. (1998) also reported some partial NifH sequences from *Azoarcus* (beta) that are in this cluster, though most *Azoarcus* NifH sequences are, more expectedly, in the gamma/beta clade. It is now clear that the phylogeny of NifH sequences within class B is not consistent with the organismal phylogeny, but there is as yet no obvious reason to exclude either gene duplication or gene transfer as the explanation.

The NifD and NifK trees (Figure 2) can be rooted using sequences for their homologs NifE and NifN respectively (see G. Fani, this volume, for more on the evolution of these four proteins). Both NifD and NifK fall into three clades corresponding to the A, B, C clades of NifH. The main discrepancy is that the vanadium nitrogenases, composed of VnfD and VnfK, are clearly in class A, whereas the corresponding VnfH reductase components (NDF2_AZOCH, NIF2_AZOVI in Figure 1) are in class B closely similar to those of the molybdenum enzymes. This suggests a relatively recent replacement of the original "VnfH" by a duplication of the *nifH* gene, as implied by Pau (1989). In class B, the NifD tree is poorly resolved, but NifK shows some unexpected relationships similar to those of NifH. Clearly our understanding of nitrogenase evolution is not yet complete.

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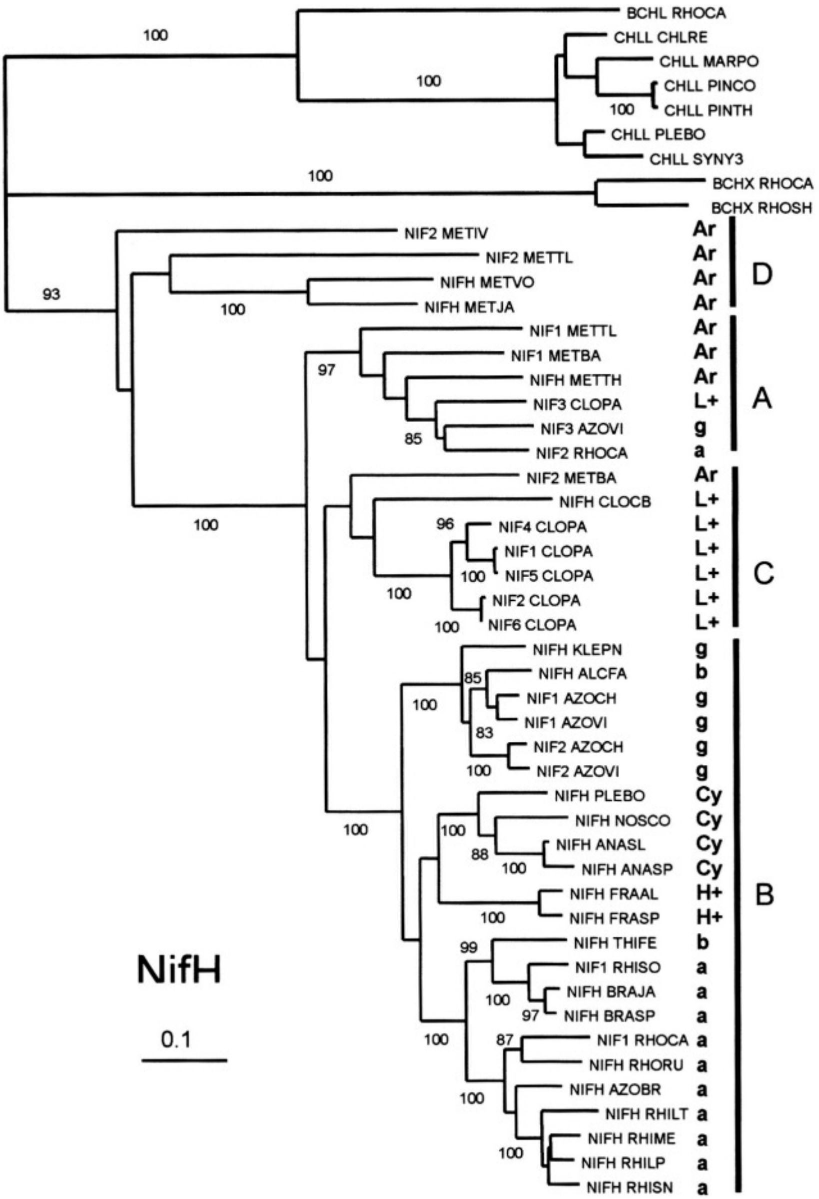
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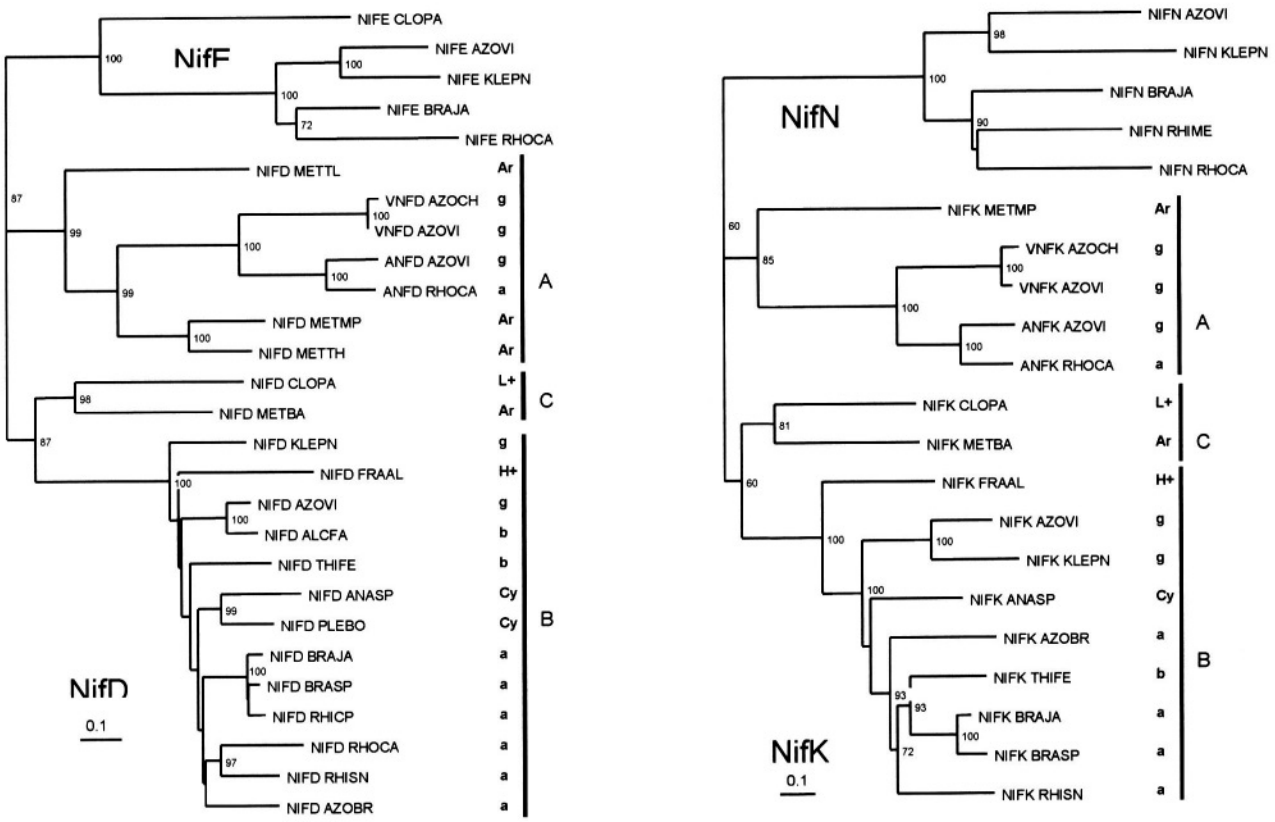
BCHX RHOSH
NIF2 METIV Ar
NIF2 METTL Ar
NIFH METVO Ar
NIFH METJA Ar
93
100
D
NIF1 METTL Ar
NIF1 METBA Ar
97
NIFH METTH Ar
NIF3 CLOPA L+
NIF3 AZOVI g
85
NIF2 RHOCA a
A
NIF2 METBA Ar
NIFH CLOCB L+
96
NIF4 CLOPA L+
NIF1 CLOPA L+
100
NIF5 CLOPA L+
100
NIF2 CLOPA L+
100
NIF6 CLOPA L+
100
C
NIFH KLEPN g
85
NIFH ALCFA b
100
NIF1 AZOCH g
NIF1 AZOVI g
83
NIF2 AZOCH g
100
NIF2 AZOVI g
NIFH PLEBO Cy
100
NIFH NOSCO Cy
100
NIFH ANASL Cy
88
100
NIFH ANASP Cy
NIFH FRAAL H+
100
NIFH FRASP H+
99
NIFH THIFE b
NIF1 RHISO a
100
NIFH BRAJA a
97
NIFH BRASP a
100
87
NIF1 RHOCA a
NIFH RHORU a
NIFH AZOBR a
100
NIFH RHILT a
NIFH RHIME a
100
NIFH RHILP a
NIFH RHISN a
B
NifH
0.1

Figure 1. Phylogeny of NifH estimated by the Neighbor-Joining method from full-length protein sequences in the SwissProt database. Sequences are identified by their SwissProt names (generally 3 characters of genus and 2 of species name, but note RHISO is *Azorhizobium caulinodans*, RHISN is *Sinorhizobium* NGR234). Taxonomic groups are identified: Ar archaea; a, b, g proteobacteria (alpha, beta, gamma); L+, H+ gram positives (low, high G+C); Cy cyanobacteria. The four major clades are identified as A, B, C, D (also called II, I, III, IV). The tree is rooted on plant and cyanobacterial protochlorophyllide synthases.


NifF
NIFE CLOPA
NIFE AZOVI
NIFE KLEPN
NIFE BRAJA
NIFE RHOCA
NIFD METTL
VNFD AZOCH
VNFD AZOVI
ANFD AZOVI
ANFD RHOCA
NIFD METMP
NIFD METTH
NIFD CLOPA
NIFD METBA
NIFD KLEPN
NIFD FRAAL
NIFD AZOVI
NIFD ALCFA
NIFD THIFE
NIFD ANASP
NIFD PLEBO
NIFD BRAJA
NIFD BRASP
NIFD RHICP
NIFD RHOCA
NIFD RHISN
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0.1
A
C
B
Ar
g
a
L+
H+
b
Cy
100
87
99
98
97
72
NifN
NIFN AZOVI
NIFN KLEPN
NIFN BRAJA
NIFN RHIME
NIFN RHOCA
NIFK METMP
VNFK AZOCH
VNFK AZOVI
ANFK AZOVI
ANFK RHOCA
NIFK CLOPA
NIFK METBA
NIFK FRAAL
NIFK AZOVI
NIFK KLEPN
NIFK ANASP
NIFK AZOBR
NIFK THIFE
NIFK BRAJA
NIFK BRASP
NIFK RHISN
NifK
0.1
60
85
81
90
93

Figure 2. Phylogenies of (a) NifD and NifE and (b) NifK and NifN. See Figure 1 for method and abbreviations.

PHYLOGENY OF RHIZOBIA

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16S rRNA sequence analyses have been used to infer the phylogenetic relationships among rhizobial species by comparison with results obtained from sequences of two alternative loci. We consider the possibility of whether a biological species approach might be useful in defining species limits in the rhizobia. However, first we consider one of the most widely used criteria currently used for estimating phylogenetic relationships among bacteria, 16S rRNA sequence homology.

Traditional classification schemes of living organisms rely upon variation in morphological characters and a fossil record. Since there are only few useful morphological characters for rhizobia, classification schemes based on variation in genetic characters often are more informative than schemes based on phenotypic variation. An advantage of this approach is that DNA sequences and gene products can be compared in an evolutionary context (molecular systematics). Usually evolutionary history (or phylogeny) is then represented as a bifurcating hierarchical tree. There is a general consensus that a bacterial species should be monophyletic, or at least approximately so, which means that most of the DNA of the members of a species should be derived from a single common ancestor.

Established opinion is that evolution throughout the genome progresses at a constant rate by mutation and Darwinian selection and that the evolutionary history of the 16S rRNA gene approximates that of the whole genome. Therefore, it became acceptable to reconstruct evolutionary relationships among bacteria from data of sequence divergence among their 16S rRNA genes. However, lateral gene transfer and intragenic recombination also are important processes by which genomes evolve. Evidence is growing that these processes must also be considered more critically in the evolution of the 16S rRNA genes.

Multiple copies of the *rrn* operons often occur in many bacteria. Within a single strain, operon organization and gene sequences may vary (Asai *et al.*, 1999; Cilia *et al,* 1996). Some genes may be nonessential and subject to homologous recombination (Jumas-Bilak *et al.*, 1998; Ng *et al.*, 1999) and environmental selection (Condon *et al.*, 1995; Cundliffe, 1990). By far the most extreme example of gene copy sequence divergence in a single wild-type strain is the 6.4% nucleotide sequence difference between two identical pairs of the 16S rRNA gene of *Thermobispora bispora* (Wang *et al.*, 1997), which also contained three identical 23S rRNA gene copies.

To evaluate the possibility that the 16S rRNA genes in some rhizobia are the progeny of lateral gene transfer and recombination events (Eardly *et al.*, 1995), we compared 16S and 23S rRNA gene phytogenies for the same set of strains. Similar tree topolgies were obtained, however, placement of species of the genus *Sinorhizobium* and *Agrobacterium* relative to the genus *Rhizobium* differed between the two reconstructed phylogenies (Fig. 1). Reconstructions of phylogenies from 23S rRNA and the Rnase P gene sequences were congruent (data not shown). Based on these results we concluded that the suggested partition of *Sinorhizobium* and *Rhizobium* into different genera (De Lajudie *et al.*, 1994) is not warranted.

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Although investigations of evolutionary relationships in rhizobia have focused more on the "fast-growing" genera, bradyrhizobial phylogeny and taxonomy is receiving some attention. Most attention has been centered on the symbionts of soybean. Initially, *Bradyrhizobium japonicum* was the only recognized species (Fred *et al.*, 1932; Jordan, 1982). Later, Hollis *et al.*, 1981 separated *B. japoncum* into three DNA homology groups, which stimulated Kuykendall *et al.*, 1992 to propose a separate species, *B. elkanii*, for one of the groups. Although for comparison phylogeny usually is compared to the type strains of *B. japonicum* (USDA 6) and *B. elkanii* (USDA 76), 17 different serogroups of soybean bradyrhizobia have been described (Date and Decker, 1965). This variation in serology may indicate that the soybean bradyrhizobia are genetically more diverse than would be apparent solely from the type strains and that comparisons leading to the proposal of *B. liaoningense* (Xu *et al.*, 1995) may have been limited. Similarly, the phylogenetic placement of the bradyrhizobia of *Lupinus* spp. (Barrera *et al.*, 1997), *Acacia albida* (Dupuy *et al.*, 1994) and *Aeschynomene spp.* (Wong *et al.* 1994) was relative to these soybean type strains.

The magnitude of the 16S rRNA gene sequence divergence among the serotype strains, *B. japonicum*, *B. elkanii* and *B. liaoningense* was limited and in many cases was insufficient to distinguish their phylogenetic placement (Fig. 2). We resolved this issue by sequence analysis of the Internally Transcribed Spacer (ITS) regions between the 16S and the 23S rRNA genes since they are less conserved than the ribosomal genes. Yoon *et al.* (1997) used a similar approach to resolve some issues of classification in *Saccharomonospora* by sequence analysis of the ITS region. From the reconstructed phylogeny based on the ITS sequences we distinguished five separate groups among the soybean bradyrhizobia. The serotype strain USDA 129 was distinct from USDA 123 and USDA 127 even though these three strains have been grouped together in the 123 serocluster, which is based on cross-reaction with antisera prepared against USDA 123 (Schmidt *et al.*, 1986). The separation of the 129 serogroup from the 123 and the 127 serogroups was supported by results from Amplified Fragment Length Polymorphism (AFLP) analyses (data not shown). The genomes of 129 serogroup strains were more similar to the genomes of 62, 110, and 122 serogroup strains than they were to the genomes of 123 and 127 serogroup strains. Besides obtaining evidence for the genetic distinction among members of the 123 serocluster, we also obtained evidence for genetic and phenotypic homogeneity between a serotype strain of *B. japonicum* and *B. liaoningense.* The strain of *B. japonicum* USDA 135 and *B. liaoningense* possess identical 16S rRNA gene and ITS sequences and *B. liaoningense* cross-reacted with anti-sera prepared against somatic cells of USDA 135. These characters conflict with the proposal of *B. liaoningense* as a species since the 135 serogroup is considered to be *B. japonicum*

Although, DNA homology currently is considered to be the most definitive approach for establishing species limits in bacteria (Stackebrandt and Goebel, 1994), Maynard Smith (1995) suggested that its use to define bacterial species is without merit because arbitrary divisions are imposed upon a continuum. As an alternative approach Dykuizen and Green (1991) suggested the possibility of applying a biological species concept, where a species might be defined as a group of strains that share a common gene pool. We used this rationale to help resolve conflicting results produced by other, more established, methods in an investigation of rhizobia isolated from the legume *Medicago ruthenica.* We considered that USDA 1877 could be a *R. etli* from an initial assessment of strong 16S rRNA gene sequence similarity with the type strain (99.7%). However, USDA 1877 could not be confidently assigned to this species because of low DNA homology with the type strain (22%). From a subsequent MLEE analysis we concluded that there may be a history of genetic recombination between USDA 1877 and other members of *R. mongolense*, even though the level of DNA homology between USDA 1877 and the type strain for *R. mongolense* was only 21%.

Therefore, we provisionally included USDA 1877 within *R. mongolense* based solely on the MLEE data. Although the limitations of a biological species approach are not clear, we suggest that the information obtained from this approach may be useful when deciding species limits in rhizobia. Until complete genome sequencing as an approach becomes readily available, it is imperative that we base our taxonomic conclusions on a multidimensional approach in attempts to define species limits, and that such an approach should consider similarities and differences at the levels of the gene, the genome, and the population.

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Figure 1. Comparison of the evolutionary history of rhizobia reconstructed from the 16S or the 23S ribosomal gene sequences. Jukes-Cantor distances were derived from aligned sequences to construct unrooted trees using the Neighbor-Joining method. The sequences were aligned using the PILEUP program in the Wisconsin package of the Genetics Computer Group (Madison, Wis.) and the aligned sequences were analyzed using MEGA (Kumar *et al*, 1993). Bootstrap values were obtained from 500 permutations of the data sets.

Figure 2. Phylogenetic relationships among the soybean bradyrhizobia reconstructed from the 16S or the Internally Transcribed Spacer (ITS) region sequences between the 16S and 23S rRNA genes. Tree construction was as in Figure 1.

Fig. 1

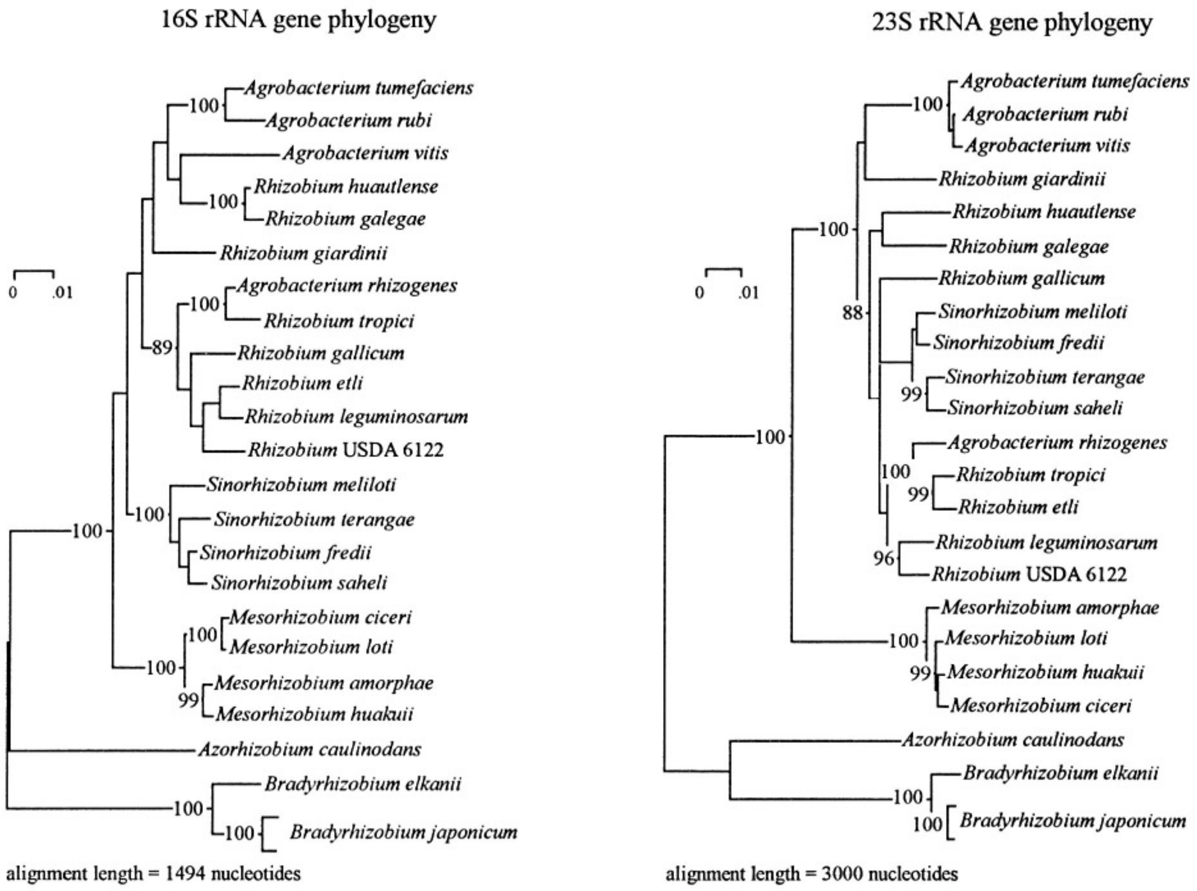
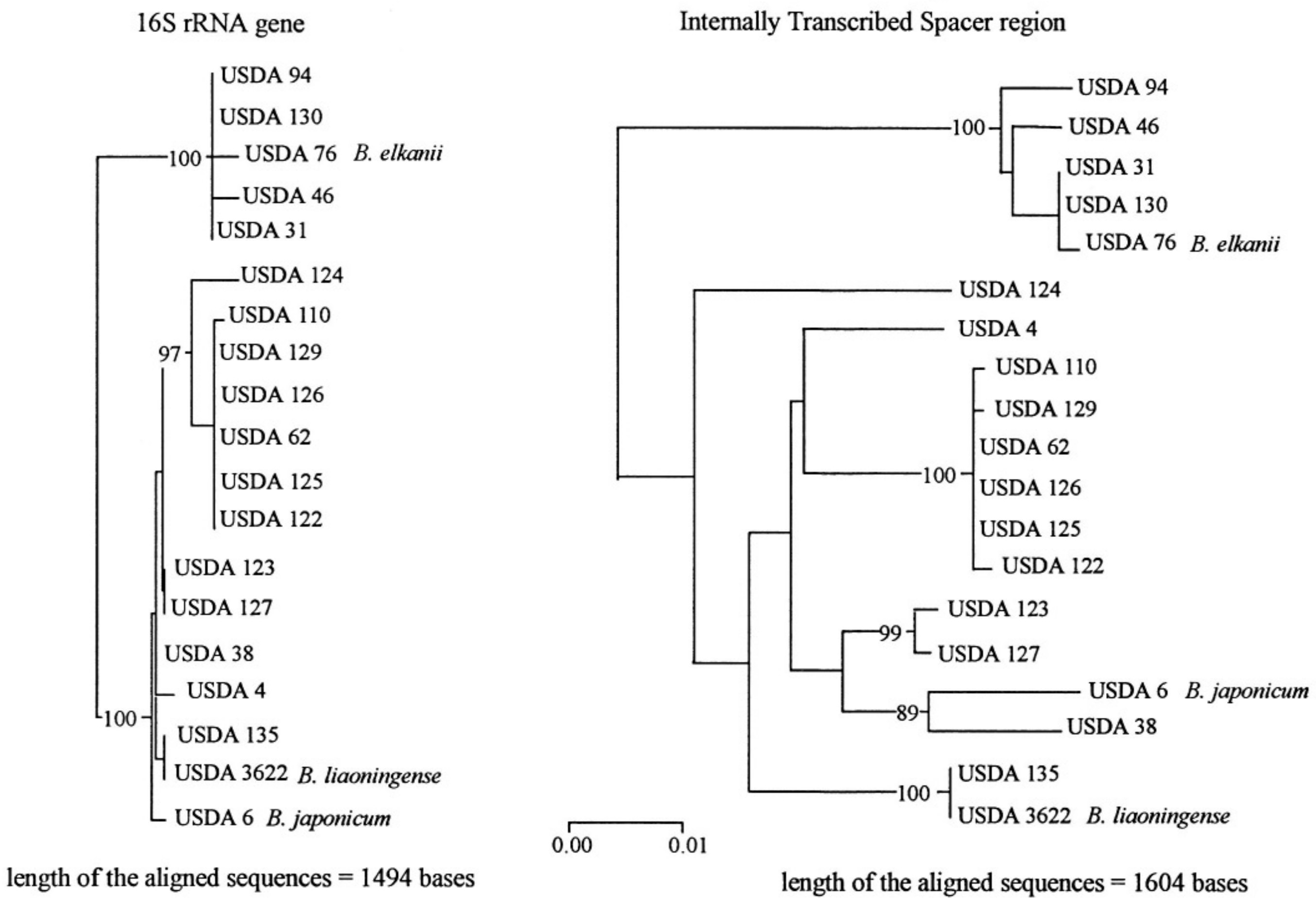

16S rRNA gene phylogeny
0
.01
100
Agrobacterium tumefaciens
Agrobacterium rubi
Agrobacterium vitis
100
Rhizobium huautlense
Rhizobium galegae
Rhizobium giardinii
100
Agrobacterium rhizogenes
Rhizobium tropici
89
Rhizobium gallicum
Rhizobium etli
Rhizobium leguminosarum
Rhizobium USDA 6122
100
Sinorhizobium meliloti
Sinorhizobium terangae
Sinorhizobium fredii
Sinorhizobium saheli
100
100
Mesorhizobium ciceri
Mesorhizobium loti
100
99
Mesorhizobium amorphae
Mesorhizobium huakuii
Azorhizobium caulinodans
100
Bradyrhizobium elkanii
100
Bradyrhizobium japonicum
alignment length = 1494 nucleotides
23S rRNA gene phylogeny
0
.01
100
Agrobacterium tumefaciens
Agrobacterium rubi
Agrobacterium vitis
Rhizobium giardinii
100
Rhizobium huautlense
Rhizobium galegae
Rhizobium gallicum
88
Sinorhizobium meliloti
Sinorhizobium fredii
99
Sinorhizobium terangae
Sinorhizobium saheli
100
Agrobacterium rhizogenes
100
99
Rhizobium tropici
Rhizobium etli
96
Rhizobium leguminosarum
Rhizobium USDA 6122
Mesorhizobium amorphae
100
Mesorhizobium loti
99
Mesorhizobium huakuii
Mesorhizobium ciceri
Azorhizobium caulinodans
100
Bradyrhizobium elkanii
100
Bradyrhizobium japonicum
alignment length = 3000 nucleotides

Fig. 2


16S rRNA gene
USDA 94
USDA 130
100
USDA 76 *B. elkanii*
USDA 46
USDA 31
USDA 124
USDA 110
97
USDA 129
USDA 126
USDA 62
USDA 125
USDA 122
USDA 123
USDA 127
USDA 38
USDA 4
100
USDA 135
USDA 3622 *B. liaoningense*
USDA 6 *B. japonicum*
length of the aligned sequences = 1494 bases
Internally Transcribed Spacer region
USDA 94
100
USDA 46
USDA 31
USDA 130
USDA 76 *B. elkanii*
USDA 124
USDA 4
USDA 110
USDA 129
USDA 62
100
USDA 126
USDA 125
USDA 122
USDA 123
99
USDA 127
USDA 6 *B. japonicum*
89
USDA 38
USDA 135
100
USDA 3622 *B. liaoningense*
0.00
0.01
length of the aligned sequences = 1604 bases

GENETICS OF *NOSTOC* IN RELATION TO HOST PLANT SPECIES

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1. Introduction

Cyanobacteria constitute an ancient group of photosynthetic prokaryotes, dating back to Precambrium, 2.8-3.5 billion years ago. Through evolution they have had a substantial influence on the biotic and abiotic environment on Earth. When cyanobacteria evolved they brought about oxygenation of the atmosphere by introducing chlorophyll *a*-based photosynthesis, leading to the production and release of oxygen to the atmosphere. Later, cyanobacteria played another fundamental role, namely in the evolution of chloroplasts. According to the endosymbiotic theory some eukaryotic progenitors merged with photoautotrophic cyanobacteria, resulting in photosynthetic eukaryotic organisms and the evolution of plants (Margulis, 1970; Douglas, 1994).

Besides performing oxygenic photosynthesis many cyanobacteria can in addition fix atmospheric nitrogen. Furthermore, they generally have a unique capacity to tolerate extreme environments (Dodds et al., 1995; Potts, 1996; Bergman et al., 1999). These features may have led to the success of cyanobacteria which is one of the most widespread prokaryotic microorganisms on Earth. They live in various habitats worldwide, from aquatic environments to terrestrial systems, in tropical soils as well as in polar regions. In addition, certain groups can form symbiosis with a variety of eukaryotic organisms such as protists, animals, fungi and plants (Rai, 1990). Cyanobacteria have thus several essential functions in the global ecosystem, being important nitrogen fixers and primary producers of organic carbon in many marine and terrestrial systems. In addition, in symbiotic associations with plants and fungi they enable the host to colonise nutrient poor habitats and hostile environments. Nevertheless, the *Azolla*-cyanobacterium symbiosis is so far the only of economical importance to farming systems, and has been used as green manure in rice field for many centuries.

2. Taxonomy

The taxonony of cyanobacteria (blue-green algae) has been quite confusing during the last few decades. This is because the taxonomic system was changed in the seventies, when it was suggested that since cyanobacteria are prokaryotes, the taxonomy should follow the Bacteriological Code (Stainer et al., 1978; Rippka et al., 1979). Previously, cyanobacteria were assigned to the algae and classified according to the Botanical Code due to their ability to perform chlorophyll- *a* dependent photosynthesis. This change from one taxonomic system to another has, and still does, create many confusions and relocation of different taxa. The basic bacteriological taxonomy of cyanobacteria was published by Rippka et al., 1979. This taxonomy relies on morphological and physiological characteristics and is widely used (see Bergey's manual of Systematic Bacteriology 1989). According to this classification, the cyanobacteria are divided into five distinct sections termed I, II, III, IV and V. Section IV contains filamentous cyanobacteria able to differentiate specialised N_2-fixing cells (heterocysts), and are in addition characterised by division in one plane only. Affiliated to this section are the following genera: *Anabaena*, *Cylindrospermum*, *Scytonema*, *Calothrix* and *Nostoc*.

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3. The Genus *Nostoc*

The genus *Nostoc* is one of the most widespread terrestrial cyanobacteria known. It is found as free-living as well as in symbiotic associations with plants. With few exceptions, symbiotically competent cyanobacteria have been identified as *Nostoc*. The genus exhibits an extremely high structural and functional plasticity, which most likely is the key to its success. Influenced by different environmental changes, *Nostoc* can undergo differentiation. When grown under nitrogen-limited conditions or in symbiosis, vegetative cells are differentiated to specialised nitrogen-fixing heterocysts being approximately 5% in the free-living stage and up to 80% in symbiosis with a host like *Gunnera* (Silvester, 1976; Bergman et al., 1992). In addition, the entire vegetative filament can differentiate into a motile stage, the hormogonium, which serves as a mean of dispersal under unfavourable conditions and constitute the "infective unit" during establishment of the symbiosis. There are strong evidence that the host secretes a compound(s) which induce this differentiation (Campell, Meeks, 1989; Gantar et al., 1993; Rasmussen et al., 1994). Finally, akinetes are differentiated under adverse conditions and function as spores. Akinete differentiation is an uncommon stage in symbiotic associations, and only known to play a role in *Azolla* where they function as cyanobacterial inocula for the next sporophyte generation (Perkins, Peters 1993). In addition, the cyanobacteria must alter their metabolism from a free-living phototrophic to a symbiotic, heterotrophic life to be symbiotically competent. The mechanisms involved in this shift is almost unknown. Recently, a new type of genome rearrangement which may be linked to the capacity of cyanobacteria to adapt to changing environments has been described (Robinson et al., 1997). It is well known that genomic rearrangements are taking place during heterocyst differentiation (Haselkorn, 1992). This extreme flexibility makes identification and taxonomy based on morphology problematic and unreliable. Furthermore, when isolated and cultured, the risk of favouring fast-growing minors or contaminants is present. This problem is well documented among isolates from *Azolla*, in which all cultured isolated symbionts are distinct from freshly isolates (Gebhardt, Nierzwicki Bauer 1991). At present, the taxonomic relatedness of the *Nostoc* symbiont is largely unknown and do not extend beyond the genus level. However, in recent years molecular methods have contributed to a more detailed classification of the genus *Nostoc*.

4. Plant Host Range and Specificity

Nostoc forms efficient nitrogen-fixing symbioses with an exceptionally broad range of representatives within the plant kingdom (Rai, 1990). This includes plants from all divisions: Bryophyta (mosses, liverworts, and hornworts), Pteridophyta (aquatic ferns of the genus *Azolla*), gymnosperms of the family Cycadaceae, and angiosperms of the family Gunneraceae, as well as diverse lichenized fungi. Not only does *Nostoc* have a broad host range, but also the host organ infected varies between the different hosts. The cyanobacteria are found extracellular in several symbioses; among the fungal hyphae in lichens, in cavities of the bryophyte thalli and of the *Azolla* leaves, and in specialised roots of cycads, whereas they are found intracellularly in the stem glands in *Gunnera*. This broad host- and tissue range places cyanobacteria in a unique position among nitrogen-fixers.

The ability to grow the two symbiotic partners separately and then reconstitute the symbiotic association under laboratory conditions (possible for the hosts *Gunnera* and bryophytes) has been used to investigate the specificity of the interaction. *Nostoc* isolated from cycads, the bryophytes *Anthoceros* and *Blasia* were found to infect *Gunnera* (Enderlin, Meeks, 1983; Johansson, Bergman, 1994). Also the work by Leizerovich et al. (1990), where different symbiotic isolates from lichens, cycads and *Gunnera* were compared with respect to restriction sites in the *nif* gene region, showed that an identical hybridisation pattern was obtained from *Gunnera* and lichen isolates. These results confirm the concept that cyanobacteria exhibit a very low host specificity with no correlation to the taxonomic status of the host. It also reflect a great adaptive flexibility of the symbiont since the same strain has the capacity to located either extra- or intracellular depending on the host.

5. Genetic Diversity

In recent years different molecular approaches such as RFLP mapping, DNA fingerprinting and sequencing of the $\mathrm{tRNA}^{\mathrm{LEU}}$(UAA) intron as well as protein profiles have been used to discriminate between different symbiotic isolates of *Nostoc* within and between different host species.

5.1. *Gunnera*

The genetic diversity of isolates from different *Gunnera* species have been studied using RFLP and PCR fingerprinting (Zimmerman, Bergman, 1990; Rasmussen, Svenning, 1998). The PCR fingerprinting method developed in our laboratory is based on the presence of repeated DNA [short tandemly repeated repetitive (STRR) and long tandemly repeated repetitive (LTRR)] sequences in the genome of heterocystous cyanobacteria (Mazel et al., 1990; Masepohl et al., 1996). The method has been shown to be extremely useful due to (i) its specificity to heterocystous cyanobacteria and that it therefore can be used on non-axenic cultures, (ii) that it can be performed on intact filaments/symbiotic tissue, and (iii) that it has a high genetic resolution. By using this method, a high genetic diversity could be demonstrated among isolates from the same and from different *Gunnera* species. However, some isolates from different species showed the same fingerprint pattern and could be grouped into three groups (Rasmussen, Svenning 1998).

5.2. Cycads

In previous studies based on morphology a correlation between different groups of cyanobacteria and the host species was suggested (Grilli-Caiola, 1980). However, reports using genetic analysis have shown a high genetic diversity among isolates from different as well as from the same cycad species (Lindblad et al., 1989, Zimmerman, Rosen, 1992; Lotti et al., 1996; Costa at al., 1999). Even different coralloid roots from a single host species were found to harbour different cyanobacteria. Nevertheless, no variation was detected within one coralloid root. Furthermore, the same cyanobacterial strain could be detected in different cycad species.

5.3. *Azolla*

The *Azolla*-cyanobacterium symbiosis differs from the other plant-cyanobacteria symbioses in the sense that the two partners never separate. The cyanobacteria remain associated with *Azolla* throughout vegetative and reproductive growth and is passed between generations through the megasporocarp. Due to difficulties in growing the symbiont on artificial media, the identification and taxonomic status of the symbiont have been questioned repeatedly. The cyanobacteria is still designated as a single species *Anabaena azollae* belonging to the genus *Anabaena.* However, based on its ability to differentiate hormogonia (one of the criteria for distinguishing *Nostoc* from *Anabaena*, (Rippka et al., 1979)), and recent molecular results indicate that the cyanobacteria shows a closer relatedness to the genus *Nostoc* (Plazinski et al., 1990). The genus *Azolla* is divided into two sections, *Euazolla* with the species; *A. caroliniana, A. mexicana, A. microphylla, A. rubra* and *A filiculoides,* and *Rhizosperma* containing *A. pinnata* and *A. nilotica.* Different molecular techniques such as RFLP mapping and PCR fingerprinting with arbitrary primers have been used to classify the cyanobacteria inhabiting the seven different *Azolla* species. So far, the different techniques used have not distinguished more than four cyanobacterial groups (Plazinski et al., 1990; Eskew et al., 1993; Van Coppenolle et al., 1995). However, using the STRR-PCR fingerprinting on freshly isolated filaments or homogenised symbiotic tissue from the seven *Azolla* species, we have now been able to discriminate between the cyanobacteria from each of the seven species (Zheng et al., 1999). Based on the fingerprint pattern, a dendogram could be constructed which shows, that the cyanobacterial isolates from the different *Azolla* species follow the taxonomy of their host *Azolla* which strongly supports the hypothesis of co-evolution between *Azolla* and the cyanobacteria.

5.4. Bryophytes

Isolates from the hornwort *Phaeoceros* were investigated using DNA fingerprinting with arbitrary primers (West, Adams, 1997). The analysed material represents cyanobacteria from individual colonies within the same thallus, as well as thalli from different remote areas. The results reveal a high diversity of *Nostoc* strains both between different thalli and within the same thallus. However, none of the examined colonies contained more than one strain. Interestingly, free-living *Nostoc* strains collected from the soil around the plants were never found in the symbiosis, although they were symbiotically competent and able to infect *Phaeoceros* and *Blasia* under laboratory conditions. This observation emphasis the problem that what can be achieved under laboratory conditions might not reflect the situation in the field, where many different factors surely influence the establishment of the symbiosis.

5.5. Lichens

The diversity of Nostoc in lichens have been studied by sequence analysis of the $\mathrm{tRNA}^{\mathrm{Leu}}$(UAA) intron (Paulsrud et al.,1998; Paulsrud, Lindblad, 1998). The results show that only one cyanobacterium is harboured in one thallus. Additionally, a low diversity was found among lichen thalli within a species, even when samples were collected from different countries no diversity was found. This indicates that the lichen symbioses are restricted in their choice of cyanobacterium to a relative small number of *Nostoc* strains in contrast to the other plant-cyanobacterial symbioses where the host are infected *de novo.*

6. Phylogenetic Studies on *Nostoc*

Diversity of symbiotic isolates of *Nostoc* have until now been investigated using methods with a high taxonomic resolution such as PCR fingerprinting, RFLP mapping and sequence analysis of the $\mathrm{tRNA}^{\mathrm{Leu}}$(UAA) intron. Both analysis of ribosomal genes and the internal transcribed spacer (ITS) region have been used for taxonomic and phylogenetic purpose of different bacterial groups including free-living cyanobacteria. These studies have so far not been applied on symbiotic cyanobacteria. Consequently, very little is known about the phylogenetic status of these strains, and criteria for discriminating between the groups below the genus level is lacking. However, according to G. Lamenti et al. (1999), the genus *Nostoc* constitute a pronounced heterogenic group. By 16S RFLP analysis of 41 axenic *Nostoc* strains including free-living as well as symbiotic isolates, a clear clustering of the isolates into distinct groups was obtained. In our laboratory, we have preliminary results based on 16S-RFLP and ITS-RFLP analysis which also indicate that the genus *Nostoc* constitute a heterogeneous group (U. Rasmussen, M.M. Svenning, unpublished). Based on these observations, the taxonomic status of the genus *Nostoc* might have to be reconsidered. However, the use of the mentioned molecular methods and sequence analysis of conserved genes in cyanobacteria will give a better understanding of the genetic relationship between the different *Nostoc* strains and give more well-defined criteria for discriminating among groups.

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ORIGIN AND EVOLUTION OF *nif* GENES

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1. Introduction

Very little is known on the origin and evolution of *nif* genes, as well as on the molecular mechanisms, such as paralogous duplication and/or domain shuffling, which might have been involved in shaping the nitrogen fixation process. Useful hints on these issues and on the connection of nitrogen fixation with other complex metabolic pathways can be inferred by the comparative analysis of the structure and organisation of *nif* and related genes in the three cell domains, Archaea, Bacteria, and Eucarya.

2. Materials and Methods

Aminoacid and nucleotide sequences were retrieved from GenBank, EMBL, and PIR databases. BLAST (Altschul et al., 1997) probing of the DNA and protein databases were performed with the BLASTP and tBLASTn programs. The ClustalW program was used for pairwise and multiple ammo acid sequences alignment (Thompson et al., 1994) using default gap penalties.

3. Results and Discussion

A detailed analysis of *nif* gene products revealed that *nifDK* and *nifEN*, encoding the α and β subunits of nitrogenase, and the two subunits of the NifEN enzymatic complex, involved in FeMo-Co biosynthesis, belong to a paralogous gene family, in that they are the descendants of a common ancestral gene by gene duplication followed by evolutionary divergence. This analysis also permitted to trace their possible evolutionary history; according to the proposed model the four genes are the result of two successive duplication events, very likely predating the appearance of the Last Universal Common Ancestor (LUCA). The first paralogous duplication event involved an ancestral gene leading to a bicistronic operon which, in turn, underwent a paralogous operon duplication event originating the ancestors of the present-day *nifDK* and *nifEN* operons. This model is consistent with both gene order and organisation of *nifDK* and *nifEN* in archaeal and bacterial nitrogen fixing micro-organisms, where the two genes of each pair are contiguous, arranged in the same order (*nifDK* and *nifEN*) and belong to the same transcriptional unit. This hypothesis is also supported by the degree of sequence similarity between the products of the four genes, and with the maximum likelihood trees constructed by using the products of the four genes from *Azotobacter vinelandii* and *Methanococcus maripaludis* (Fani et al., 1999).

These data established a link between two of the main theories proposed to explain the origin and evolution of metabolic pathways, the retrograde hypothesis (Horowitz, 1945) and the so-called patchwork hypothesis (Jensen, 1976). According to the former, genes involved in the same metabolic pathway might have been the descendants of a single ancestral gene by in tandem paralogous duplication followed by evolutionary divergence. Jensen (1976) proposed that an ancestral pathway may have had a primitive enzyme catalysing two or more similar reactions on related substrates of the same metabolic route and whose substrate specificity was refined as a result of later duplication

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events. The model proposed for the origin and evolution of *nifD*, *K, E* and *N* may also fit the Jensen's hypothesis, in that the ancestor of the *nif* family might have encoded for a protein which might assemble to give a homomultimeric complex with a low substrate specificity with the ability to catalyse more than one enzymatic reactions. The function performed by this primordial enzyme might have depended on the composition of the early atmosphere.

A further comparison of the products of the four genes with enzymes involved in other metabolic pathways revealed that they exhibited a significant degree of sequence similarity to the products of *Rhodobacter capsulatus*, *R. sphaeroides*, and *Heliobacillus mobilis bchB* and *bchN* genes, which encode for two components of protochlorophyllide reductase, and the products of *bchY* and *bchZ*, encoding two components of chlorin reductase. The two enzymes catalyse two sequential steps of bacterioclorophyll biosynthesis. It has been previously shown that *bchB* and *bchY* are paralogous genes, as well as *bchN* and *bchZ.* Data obtained strongly suggested that *bchlB*, *bchlN, bchlY,* and *bchlZ* genes belong to the *nifDKEN* paralogous family, suggesting that they might have arisen from duplication of one of the two operons above mentioned. This in agreement with the previous idea of the connection existing between nitrogen fixation and bacterial photosynthesis. In fact Burke et al (1995) demonstrated that the *nifH* gene product exhibited a very high degree of sequence similarity to the third component of protochlorophyllide reductase and chlorin reductase, encoded by *bchlL* and *bchX*, respectively, suggesting that the latter genes were the result of a paralogous duplication of *nifH.* We proposed a model were the pair *bchNB* was the result of a paralogous operon duplication of *nifDK* or *nifEN* operon; *bchNB* in turn gave rise to *bchYZ* by a second paralogous operon duplication event.

These data suggested that, during the early stages of molecular evolution, paralogous duplication of DNA stretches have played an important role in generating genes or operons encoding proteins with similar enzymatic activities and in shaping nitrogen fixation and bacteriochlorophyll biosynthesis.

This is also confirmed by data concerning the common origin of *nifV* and *leuA; nifV* encodes an homocitrate synthase catalysing the condensation of acetyl-coA and α-ketoglutarate giving rise to homocitrate. The product of *leuA* encodes an α-isoproprilmalate synthase, which carries out the condensation between acetyl-coA and a-ketoisovalerate and is the first enzyme of leucine biosynthesis in bacteria, fungi and plants. The comparative analysis of the products of these two genes from organisms belonging to the three cell domains revealed that they share a very high degree of sequence similarity and that they are paralogous. We suggest that both of genes were part of the genome of the last universal common ancestor and that duplication event of the common ancestral gene occurred before the appearance of the cenancestor.

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CHARACTERIZATION OF NITROGEN-FIXING BACTERIA CONTAINING MOLYBDENUM-INDEPENDENT NITROGENASES FROM DIVERSE NATURAL ENVIRONMENTS

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1. Introduction

Azotobacter vinelandii, an aerobic diazotroph was the first soil nitrogen-fixing bacterium shown to have three genetically distinct nitrogenases; nitrogenase 1, nitrogenase 2, and nitrogenase 3 (Bishop and Premakumar, 1992). Nitrogenase 1 is the classical molybdenum (Mo)-containing nitrogenase and is expressed in N-free media containing Mo. The other two are Mo-independent nitrogenases. Nitrogenase 2 is a vanadium (V)-containing nitrogenase and is expressed in Mo-deficient N-free medium containing V. Nitrogenase 3 is an iron-only nitrogenase and is expressed in Mo- and V-deficient N-free medium. Phylogenetically and physiologically diverse laboratory microorganisms have been shown to have Mo-independent nitrogenase systems. Among these are *Clostridium pasteurianum* (Zinoni F et al., 1993), *Rhodobacter capsulatus* (Schneider et al., 1991), *Anabaena variabilis* (Thiel, 1993), *Rhodospirillum rubrum* (Lehman and Roberts, 1991), *Heliobacterium gestii* (Kimble and Madigan, 1992), and *Azospirillum brasilense* (Chakraborty and Samadar, 1995). Recently our laboratory reported the isolation of seven diazotrophs with Mo-independent nitrogenases from aquatic environments using Mo-deficient, N-free enrichment media (Loveless et al., 1999). In this study we extend these results to recently isolated diazotrophs from wood chip mulch and from sediments of mangrove swamps in Puerto Rico.

2. Material and Methods

All procedures were conducted as described previously (Loveless et al., 1999) except that the Mo-deficient enrichment medium (Burk medium) contained 2% glucose instead of sucrose.

3. Resultados

Twelve diazotrophs with Mo-independent nitrogenases were isolated from wood chip mulch and from a mangrove swamp using Mo-deficient N-free enrichment media. These isolates were characterized with respect to their phylogenetic and nitrogen fixation properties. Phylogenetic analysis of 16S rRNA gene sequences indicates that all of the isolates are members of the gamma subdivision of the class *Proteobacteria* and they appear to be specifically related to the fluorescent pseudomonads. Ten of the isolates grew diazotrophically in N-free liquid media in the presence of Mo or V and in the absence of these metals. Based on Southern hybridization analysis all of the isolates have *nifD* (nitrogenase 1), 10 have *vnfD* (nitrogenase 2) and 6 have *anfD* (nitrogenase 3) genes. Except for two strains (NC-1 and NC-2), there is a good correlation between the ability to detect the required nitrogenase genes and the ability to grow under each of the three different nitrogen-fixing conditions (+Mo, +V, and -Mo) used in this study. We were unable to detect either vnfD or anfD, even after repeated attempts. However, both strains grew well under Mo-deficient conditions in the presence or absence of V.

In conclusion, we have demonstrated that diazotrophs with Mo-independent nitrogenases can be readily isolated from diverse environmental sources using Mo-deficient, N-free enrichment media.

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Since the strains reported in this study were isolated under aerobic conditions, one of our future goals will be the isolation of strains under anaerobic conditions. The results of these studies should add to our knowledge of the distribution and diversity of diazotrophs with Mo-indpendent nitrogenases in natural environments, and they may also provide clues as to the importance of these organisms to the nitrogen cycle.

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BIODIVERSITY OF RHIZOBIA FROM A WIDE RANGE OF FOREST LEGUMINOSAE SPECIES IN BRAZIL

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Rhizobia and Leguminosae Symbiosis in Brazilian ecosystems - Leguminosae is a important family in Brazilian ecosystems both in number of species and individuals. To date 1294 species are reported to occur in Amazon (Silva et al., 1989) whereas 555 species are found in the Brazilian Cerrado (Kirkbride Junior, 1984). Estimates for Atlantic forest are only available to scattered areas, but show that Leguminosae diversity is also high there. From the most intact forests to the most disturbed ones, frequencies of Leguminosae in relation to the total number of species are usually high (Table 1). It is important to point out that a high number of liana species are also found at forest ecosystems. Although the liana species are highly ignored, many of them have economic potential and are able to establish symbiosis with rhizobia (e.g. *Derris* spp.), and, therefore they will be considered here, besides trees, as also forest species.

Since Allen & Allen's (1981) report on absence of knowledge about nodulation capability of the majority of tropical Leguminosae species, intensive surveys were conducted throughout the world, mainly in Brazil (Faria et al., 1989; Moreira et al, 1992). Total number of species analysed increased from 15 to 23 %, i.e., 3856 species, from which 3397 (88%) do nodulate (Faria et al., 1999). Nodulating species are mainly in the Mimosoideae (90%) and Papilionoideae(96%) sub-families, and represents only 24% of Caesalpinioideae species. It can be estimated based on those figures, that, rhizobia in symbiosis with around 11 200 leguminous species are completely unknown around the world.

Rhizobia biodiversity - Despite the great number of rhizobia symbiosis already known (3397), taxonomic studies with the microsymbiont has been restricted to a few grain species. Only recently, rhizobia species (*Azorhizobium caulinodans, Sinorhizobium saheli, S. teranga, Rhizobium hautlense* and *Mesorhizobium plurifarium*) were described based on isolates from forest species. From these, only *M. plurifarium* includes Brazilian isolates from *Leucaena* spp and *Chamaecrista ensiformis.* The former is a highly promiscuous genera also capable of nodulating with native strains belonging to *R. tropici, R. leguminosarum, S. fredii* and *S. medicae* (Moreira et al., 1998). A large number of strains has been isolated from several forest species in Brazil. Considering collections made by CNPAB-EMBRAPA (Rio de Janeiro), INPA (Amazonas) and UFLA (Minas Gerais) there is about 4,000 strains isolated from species belonging to around 60 native genera of Leguminosae, i.e., half of the estimated number of the native nodulating genera, and from around 10 exotic genera. Strains with slow (SG) or very slow growth (VSG) (less frequently) which alkalinises YMA (Vincent, 1970), with morphological characteristics typical to *Bradyrhizobium* spp. were isolated from 89% Caesalpinioideae genera and from 70 and 76% of genera in Mimosoideae and Papilionoideae, respectively (Moreira et al., 1991). Partial 16 S rRNA sequences of 22 SG strains, isolated from different phylogenetic branches of Leguminosae, were highly similar to *B. elkanii* or *B. japonicum* (Table 2). Probably the VSG strains belong to *B. liaoningense,* which must be confirmed. Other phenotypic (SDS-PAGE protein profiles) and genotypic (multilocus enzyme electrophoresis/MEE, RAPD fingerprints) analyses on part of these SG and VSG alkanisers confirm their taxonomic position in the genera *Bradyrhizobium* (Moreira et al, 1993; Moreira et al., 1995; Coutinho et al., 1995). This result is conflicting with previous reports that *Bradyrhizobium japonicum* was introduced in Brazil as bacterial inoculants for soybeans (Martinez Romero and Caballero-Mellado, 1996). Considering that the major part of forest strains belonging to *B. japonicum* were isolated from nodules collected in natural ecosystems, present results suggest the endemism of this species.

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An introduced species should exhibit a high competitive ability, an exceptional adaptability to diverse edaphic environments and a promiscuous behave in relation to a high genetic diversity of hosts. In the case of *B. japonicum,* all these should be happened since the first inoculant strains for soybeans were introduced 60 years ago in Brazil. The wide distribution of symbiosis with *Bradyrhizobium* within almost all nodulating phylogenetic branches of Leguminosae in tropical forests suggests this symbiosis was established earlier than the others. Strains with intermediate growth were also isolated from forest species, but with much lower frequency in native genera (11% Caesalpinioideae, 30% Mimosoideae and 15% Papilionoideae). Fast growers were found in symbiosis with 44% of Caesalpinioideae and 39% of Papilionoideae genera but they are highly frequently among Mimosoideae (67%) mainly due to *Mimosa* and *Acacia* species symbiosis. *Rhizobium, Azorhizobium, Sinorhizobium* and *Mesorhizobium* species were identified among these isolates (Table 2). About 20 plant genera nodulates both with slow and fast growers. Results of partial 16S rRNA partial sequences, total protein profiles (SDS-PAGE), MEE and also the widespread *Bradyrhizobium* symbiosis, indicate no relationship between rhizobia and Leguminosae phylogenies. However, about only one third of species and half of genera of Leguminosae were analysed for nodulation and some microsymbiont phenotypic characteristics and few strains were characterised genetically. Therefore, indigenous rhizobia biodiversity is still poorly understood.

Table 1: Frequency of total and nodulating Leguminosae species in relation to the total species number of other families in 32 vegetation surveys of forest ecosystems in Brazil (Compiled from various by Pereira et al., 1998)

State/ Surveys´s date	Leguminosae species frequency range (%)	
	Total	Nodulating*
Amazonas (1971-1987)	8,9 – 25,9**	4,2 – 11,7
Pará ((1987)	13,1 – 20,8**	nd
Maranhão (1993-1996)	13,4 – 18,1**	4,8
Rondônia (1987)	15,8 – 16,9**	9,0
Minas Gerais (1992-1994)	9,8 – 19,7**	7,4 – 14,6
Espirito Santo (1992)	12,9 **	7,7
São Paulo (1992-1996)	3,6 – 27,5 **	2,8-16,7
Paraná (1996)	20,0	15,7
Rio Grande do Sul (1992)	18,3 – 22,2**	13,3-15,9
Distrito Federal (1996)	20,7	13,5

* Considering potentially nodulating species.

** Leguminosae was the family with the highest species diversity.

Table 2: Phylogenetic affinity of the partial 16 rRNA sequences. For each of the 15 different sequences obtained from 44 brazilian rhizobia strains isolated from forest species, the most similar published sequence is shown (adapted from Moreira et al,1998)

Most similar published sequence	Leg.groups*	Difference**
Bradyrhizobium japonicum ATCC10324^T	C1,M1,M3, M4,P3,P5	2
Bradyrhizobium elkanii USDA 76^T	C1, M3, M4,P2, P3, P4, P7	0
Bradyrhizobium japonicum ATCC10324^T	M4	0
Bradyrhizobium BTAi1	P7	0
Bradyrhizobium japonicum ATCC10324^T	M4	3
Azorhizobium caulinodans ORS 571^T	P6	8
Rhizobium tropici CIAT 899^T	C1, P1, P3	0
Rhizobium tropici CIAT 899^T	M2	4
Agrobacterium rhizogenes IAM13570^T	M2, M4, P5, P6	0
Rhizobium leguminosarum ATCC 10004^T	P6	2
Rhizobium leguminosarum ATCC 10004^T	M2	3
Sinorhizobium fredii USDA 205^T	M2	0
Sinorhizobium medicae A 321^T	C1, M2, M4, P1	0
Sinorhizobium sp. (Prosopis) HAMBI 1393	M2	0
Mesorhizobium huakuii CCBAU 2609^T	C3	0

*Respectively, in C=Caesalpinioideae , M=Mimosoideae, P=Papilionoideae.**Number of nucleotides

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BIODIVERSITY AND SELECTION OF *SINORHIZOBIUM FREDII* STRAINS FOR SOYBEAN INOCULANTS.

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The bacteria able to nodulate and establish an efficient symbiosis with soybean [*Gycine max* (L). Merrill] are classified as *Bradyrhizobium japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall et al. 1992), *Sinorhizobium fredii* and *S. xinjiangensis* (Chen et al. 1988). Keyser et al. (1982) were the first to report on the isolation of *S. fredii* from root nodules and soils collected in the People's Republic of China. Since then, several groups have reported the isolation of new *Sinorhizobium* strains from Malaysia, Vietnam and Brazil (Cleyet-Marel, 1987; Young et al., 1988; Chueire and Hungria, 1997).

The isolates reported by Keyser et al (1982) form effective nitrogen fixing symbioses with non-bred soybean cultivars as Peking and Malayan (Keyser et al., 1982; Ruíz-Sainz et al., 1984), but they are generally ineffective with North American soybean cultivars. This fact set forth the notion that the value of *S. fredii* strains as commercial soybean inoculants was very limited. After these pioneer studies, it has been largely demonstrated that the symbiotic potential of *S. fredii* strains with Western soybeans is similar to that of *B. japonicum* strain USDA110 (Dowdle and Bohlool, 1985; Balatti and Pueppke, 1992; Buendía-Clavería et al., 1994; Rodriguez-Navarro et al., 1996; Chueire and Hungria, 1997). Numerical taxonomic studies have set up the taxonomic position of this new rhizobia group. We have studied two different sinorhizobia collections, from China and Brazil. In both cases, bacterial strains were isolated from nodules induced on different soybean (non-bred and modern) genotypes after plant inoculation with soil samples. Chinese soils were predominantly alkaline (pH 8.0), they harbored a native rhizobia population (10^4-10^5 bacteria per gram of soil), and have many years of soybean cultivation. In contrast, Brazilian soil samples were acidic (pH 3.0-5.0) and bacteria came from undisturbed soils with no record of soybean cultivation and from soybean cropping areas, thus containing a naturalized population of soybean bradyrhizobia that was introduced as inoculants in the past decades.

A numerical taxonomic study of these sinorhizobia collections is being undertaken through the analyses of the following parameters: symbiotic properties, colony morphology, acidification of YM medium, tolerance to extreme conditions (acidity, alkalinity, salinity, high temperature), serological studies, growth with different C and N sources, intrinsic antibiotic and heavy metal resistance, plasmid, LPS and protein profiles, genetic analyses with ERIC and REP-PCR.

Some outstanding features can be drawn: the trap-host cultivar/native rhizobia interaction is an important factor to be considered during the estimation of the native population size by using the MPN technique. The plant genotype used as trap-host can also affect the balance of fast- and slow-growers isolated from a particular soil. The general trend is that modern soybean cultivars would preferently trap slow-growers (*B. japonicum*), while non-bred or Asiatic cultivars would mostly trap fast-growers (*S. fredii*).

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The pH at which the soybean plants are growing can influence the percentage of nodules formed by fast- and slow-growers, as well as the competitive ability of each type of soybean microsymbionts. Usually, a higher number of slow-growers are isolated at low pH, while fast-growers are preferentially isolated at alkaline pH. Results also indicate that *B. japonicum* could be more competitive than *S. fredii* in acid conditions while fast-growers strains could outcompete slow-growers in alkaline soils.

In Spain, in soils with high pH, field trials have been carried out to access the symbiotic potential of *S. fredii* inoculants with several modern soybean cultivars (Williams, Kure, Osumi and Ardir). The results have shown that *S. fredii* inoculants produced a seed yield (kg/ha) and a seed N content that were not significantly different from those obtained by USDA 110 or by N-fertilized (200 kg N/ha) plants.

In Brazil, fast-growers were present in 12 out of the 22 soils analyzed and 30 isolates were obtained. In cropped soils, the fast-growers represented 14% of the soybean rhizobial population, while in undisturbed soils, they represented 17% of the population. Although most of the isolates were trapped by non-bred soybean genotypes, reinoculation experiments confirmed that all fast-growing strains formed effective **N_2-fixing** nodules with both non-bred and modern soybean genotypes. However, in field experiments performed with the modern cultivars BR-16 and BR-37, in acidic soils with an established population of soybean bradyrhizobia, a poor performance of the fast-growing strains was observed, with most of the nodules being occupied by the slow growers.

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TAXONOMY OF THE ACETOBACTERACEAE FAMILY

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The Acetobacteraceae includes acetic bacteria that occur in different vegetal products and colonize the surface and internal parts of plants. According to the SSU rDNA sequences of the currently known members of the α-Proteobacteria, the Acetobacteraceae is located in a branch with close relation to the family Rhodospirillaceae (*Azospirillum* and relatives). As in other α-Proteobacteria, nitrogen fixing strains have been identified in the Acetobacteraceae. N_2-fixing species, like Rhizobia, Rhodobacters and Bradyrhizobia, are located in more distant branches of the α-Proteobacteria (Ludwig et al, 1998).

The taxonomy based on the ribosomal gene sequences of the Acetobacteraceae species has shown almost complete agreement with the traditional taxonomy of this family. The currently recognized genera in the Acetobacteraceae are *Acetobacter* and *Gluconobacter* (Figure 1). Based on the type of prevalent producing ubiquinone and on the SSU rDNA sequence analysis, Yamada et al. (1997) proposed to split the genus *Acetobacter* into two clusters, one with a new genus name *Gluconacetobacter* (*Gluconoacetobacter* [sic]) for those species that synthesize ubiquinone Q_{10}, excepting *A. methanolicus,* and the other, with the former name, *Acetobacter,* for the ubiquinone Q_9-possessing group, with species that show the highest relation with *A. aceti.* From the SSU rDNA tree it is noticeable that the family Acetobacteraceae is composed by different clusters. One of them corresponds to the genus *Gluconobacter.* The other one contains a single species, *A. methanolicus.* Two other clusters are the ones that have been proposed to be relocated to *Gluconacetobacter,* one of that clusters is composed by species related to *A. liquefaciens* and the other one with species related to *A xylinus.* The proposal of the new genus, *Gluconacetobacter,* needs to be corroborated by data from other gene sequences. In *A. diazotrophicus* we have documented the existence of several copies of ribosomal genes (Caballero-Mellado et al., 1995) and recombination events that have been shown in multicopy ribosomal genes in other bacteria may cause distortion in phylogenies based on ribosomal genes.

The only N_2-fixing species recognized so far in this family, *A. diazotrophicus* is located in the same cluster of *A. liquefaciens.* We have isolated and characterized N_2-fixing Acetobacteraceae strains that show phenotypic and SSU rDNA sequence differences with *A. diazotrophicus* (Jiménez-Salgado, 1997; Caballero-Mellado et al., 1999). By SSU rDNA sequence, these strains are located in the same cluster as *A. diazotrophicus* and *A. liquefaciens.* The type strain of one of the new groups, CFN-Cf55^T, shows a SSU rDNA similarity of 98.47 % to *A. diazotrophicus* PAl5^T. The type strain of the other group, CFN-Ca54^T, shows a similarity of 95.25% to *A. diazotrophicus* PAl5^T. The DNA homologies of the strains CFN-Cf 55^T and CFN-Ca54^T with *A. diazotrophicus* PAl5^T are of 19 and 12%, respectively. With the aim of designing SSU rDNA-based oligonucleotides that could be useful in rapid identification of the strains related to CFN-Cf55^T and to CFN-Ca54^T we aligned the known SSU rDNA sequences of the species of Acetobacteraceae. In the SSU rDNA genes there are three regions that could be candidates for designing taxonomic oligonucleotides in this family. We designed primers that differentiate the *A.*

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diazotrophicus, the **CFN-Cf55^T-** and the **CFN-Ca54^T-like** strains from all other Acetobacteraceae species described so far.

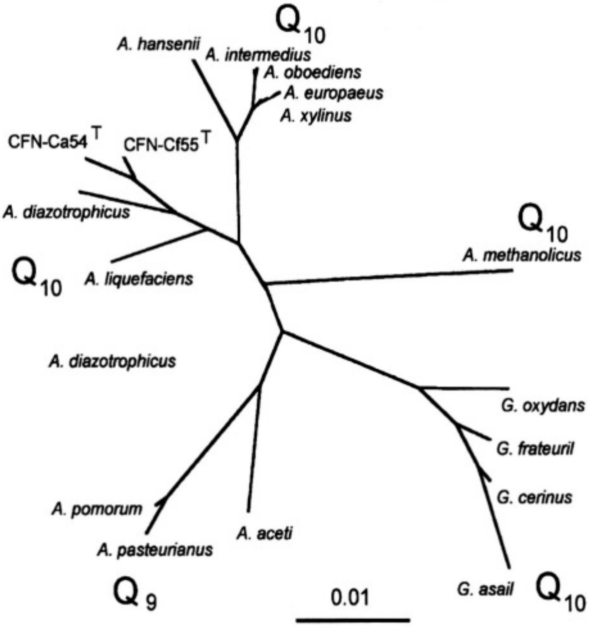
Q10
A. hansenii
A. intermedius
A. oboediens
A. europaeus
A. xylinus
CFN-Ca54 T
CFN-Cf55 T
A. diazotrophicus
Q10
A. methanolicus
Q10
A. liquefaciens
A. diazotrophicus
G. oxydans
G. frateurii
G. cerinus
A. pomorum
A. pasteurianus
A. aceti
Q9
0.01
G. asaii
Q10

Figure 1 Phylogenetic relationships of the Acetobacteracea family based on SSU rDNA

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DIVERSITY IN THE BEAN NODULATING RHIZOBIAL POPULATION OF NORTH WEST OF ARGENTINA

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1. Introduction

The southern Andes in Argentina is considered to be part of the South American centre of bean domestication. The other centre of origin took place in Mesoamerica. In the NWA region it is possible to find the wild bean *Phaseolus vulgaris* var. *aborigineus* , the ancestors of bean cultivated bean varieties, growing in virgin lands, therefore it is an interesting ecosystem to assess whether the wild bean variety had developed specificity that ended in some particular host-rhizobium association. Another interesting feature of NWA is that the Argentinian bean production resulting of 245,000 Ha of cultivated beans is generated in this region.

It had been demonstrated that *P. vulgaris* is a permissive host, and that several species have been distinguished and new species have been recently described for strains recovered from nodules collected at different sites from all over the world. The majoritiy of isolates from nitrogen fixing nodules of *P. vulgaris* in Mesoamerica are *R.etli,* whereas *R.tropici* have also been isolated from diverse South American regions such as Brazil, Colombia and also in France and Kenyan acid soils. Two additional species *R.gallicum* and *R.giardinii* have been described.

It seems important to gain more information on the occurrence of *Rhizobium* species that nodulate beans in different locations of the NWA. We examined a collection of rhizobial isolates from wild beans growing in virgin lands, and rhizobia that were retrieved from soil in laboratory using common beans and leucaena as the trapping hosts, by using analysis of chormosomal DNA markers such as RFLP-16S rDNA and rep-fingerprinting, and of symbiotic plasmid markers such as RFLP of *nod*C and *nif* H.

2. Experimental procedures

Nodules were surface sterilized with ethanol and hydrogen peroxide, and isolated axenically on YEM-Red Congo medium. Soil isolates were recovered from nodules of plants of common beans or leucaena, that were grown in the laboratory, after inoculation with soil suspension (Aguilar et al., 1998). DNA template for PCR was prepared by using resine chelex-100 in a rapid method described by Alippi and Aguilar (1998). Species assignment of isolates was done by RFLP analysis of PCR - generated 16S rDNA according to the procedure described by Laguerre et al. (1994). The procedure described by de Bruijn was used to generate rep-fingerprinting by using primers REP or ERIC, and dendrograms were obtained by using the computer-assisted system of analysis GelCompar (Applied Maths, Kortrij, Belgium). Variability of symbiotic genes was examined by RFLP analysis of PCR amplified *nod*C and *nif*H, respectively.

3. Results and Discussion

The isolates were obtained from bean collected at different altitudes in a region extended between coordinates 22° 15′ and 27° 21′ latitude S and 64°.40' and 66° 20′ longitude W. Two hundred and eleven isolates obtained from wild beans and 120 isolates obtained from the trapping hosts common beans and leucaena, respectively, were examined. It was found that 64% of isolates of wild beans had a restriction pattern of 16S rDNA identical to *R.etli* while the rest had a pattern identical to

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R.leguminosarum bv. *phaseoli.* Therefore, the wild bean-nodulating rhizobia were found to belong to the type I bean rhizobia but the clear predominancy of the *R.etli* 16S rRNA allele confirms our previous finding obtained with a limited number of isolates (Aguilar et al., 1998). Interestingly, several isolates, from different sites of NWA, had the *R.leguminosarum* 16S rRNA allele. Intraspecies diversity was demonstrated by DNA fingerprinting and RFLP of the 16S-23S rDNA intergenic region. The molecular characterization of the symbiotic genes *nif*H and *nod*C revealed different degree of diversity. It was found by RFLP patterns and amplification of the 3'- coding region of *nif*H (Aguilar et al., 1998), to be highly conserved among the *nif*H sequences of *R.etli* and *R.leguminosarum* bv.*phaseoli* strains. However, by using six different restriction enzyme in RFLP analysis of *nodC* gene, it was found four different patterns (Figure 1). Three of them were shared by both species, whereas one pattern was found only in *R.leguminosarum* bv.*phaseoli.* Two RFLP-*nodC* patterns were identical to the patterns found in the lineages represented by the *R. etli* strains CFN42 and Viking, respectively. Our results demonstrate a limited diversity at the level of species, however fingerprinting and analysis of *nodC* revealed a great intraspecies diversity. The isolates from common beans collected in cultivated areas were found to have the *R.etli* 16S rDNA allele. These results are in agreement with the size of the population type I of bean nodulating rhizobia (e.g. 10^5-10^6 rhizobia g^{-1} of soil). Two rhizobial populations were found associated with the perennial wild bean *Phaseolus augusti,* which shares the habitat with *P.vulgaris* var.aborigineus.The two 16S rRNA alleles were found similar to *B.ekanii* and *B.japonicum* USDA59, respectively.

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5. Acknowledgements

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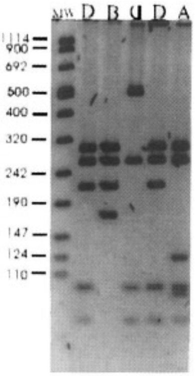

Figure 1. RFLP analysis of *nodC* of rhizobia isolates. The pattern was obtained by using *Hinf*I.

CHARACTERIZATION OF *RHIZOBIUM LUPINUS* FROM NEAR THE PARANA RIVER (ARGENTINA) BY PCR-RFLP

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1. Introduction

The lupin is grown as an alternative grain legume. It has the potential to supply a direct on farm source of protein to substitute for soybean meal specially when meal prices are high or where soybean production is marginal (López-Bellido et al., 1986). *Lupinus albescens* Hooker & Arnnott, is native in Northeastern Argentina, Paraguay and Uruguay. The *Lupinus gibertianus* C.P. Smith complex represents a group of annual lupins occurring in Brazil, Uruguay and Argentina. They has been found frequently on sandy soils an dunes near the Parana River and in its islands.The nitrogen-fixing symbiotic bacteria of lupins has been attributed to either of both genus: *Rhizobium* or *Bradyrhizobium.* This work is oriented to molecular characterization by PCR-RFLP analysis of 16S rDNA of the 30 native strains isolated: 20 belonging to the cited *Lupinus* species and 10 to *Adesmia punctata. Both legumes colonize the same geographic region*

2. Results

The 16S rDNA genotypes obtained from the unclassified analyzed *Rhizobium* strains were compared with strains from known species. This analysis of the 30 isolates revealed the existence of seventeen (17) well-defined groups, 1,2,3,..., 17 indicating intra-gene diversity within the collection. The strains isolated from *L. gibertianus* plant nodules formed a well-defined cluster in the dendogram, suggesting they are belonging to different *Rhizobium* strains. The strains isolated from *A. punctata* showed identical restriction patterns for all tested Ez, suggesting that the same strain was isolated. The *Rhizobium* restriction patterns from *Lupinus albescens* in combination with the used reference strains, produced a coherent *"Rhizobium spp."* cluster at a linkage level (S_D) of 70%, similar results were obtained by Vinuesa *et al.* (1998) with the genotype characterization of *Bradyrhizobium* strains that nodulate endemic wood Legumes as well as by our group (Fulchieri *et al.,* 1999) with characterization of *R. Loti* in the Argentine Salado River Basin.

We examined both the reinoculation with *La 4* and *La7* strains on *L. albescens* and with *Lg1* and *Lg4* on *L. gibertianus.* Plants were taken out sixty days after sowing (150-180 mm. of total length), at that time an abundant nodulation was observed (average: 9 nodules/plant). It's infectivity was demonstrated by the observed colour (presence of leghemoglobin).

3. Conclusion

The obtained characterization results from the investigation of 30 isolates, indicate the existence of 17 possible native *Rhizobium* genotypes, that potentially could form infective nodules with native *Lupinus* originating from Paraná, Santa Fe. Argentina. It is recommended to perform an exhaustive analysis of the 16S rDNA sequence in order to obtain a complete identification of the isolates.

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NITROGEN-FIXING BACTERIA ISOLATED FROM FOREST SOILS IN FINLAND

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Conifers dominate in the mature Finnish economically managed forests. The raw material demand of pulp and paper industry has recently shifted towards hardwoods. Birch also grows more rapidly than spruce in the boreal climate. For these reasons former conifer stands are often planted with birch after harvesting. Root surface bacteria of the herbaceous plants growing on the forest floor may serve as a microbe pool in early phases of tree development.

Nitrogen-fixing bacteria were isolated from acid conifer forest soils in southern Finland. The samples were taken from the humus layer of two Norway spruce (*Picea abies* L.) stands and enrichment procedure in N-free malate medium was used for the isolation. Acetylene reduction assay was performed. Genomic fingerprinting of the nitrogen-fixing pure cultures by rep-PCR produced thirteen different patterns. A representative of each was identified by fatty acid methyl ester (FAME) analysis and by 16S rDNA sequencing. Two isolates were similarly identified as *Rhodococcus erythropolis* and *Rhodococcus fascians* with the two methods. Two other isolates showed high sequence similarities to *Pseudomonas* and *Phyllobacterium,* respectively. Seven spore-forming isolates were closest to *Paenibacillus durus* (*P. azotofixans*). Amplification with *Paenibacillus* specific PCR confirmed the isolates to belong to *Paenibacillus.* The two remaining isolates belonged to γ-proteobacteria and were closest to *Yersinia* and *Stenotrophomonas* or *Xanthomonas.* The results of the sequence analysis of 16S rDNA and FAME were not in agreement in most cases. One reason might be the slow growth of the isolates in TSA, the standard medium used for the identification by the MIDI library.

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF SOYBEAN SINORHIZOBIAL STRAINS ISOLATED FROM BRAZILIAN SOILS

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Soybean [*Glycine max* (L.) Merrill] is considered one of the oldest crops in the world. *Rhizobium* strains that induce nodule formation in this plant are diverse. Brazilian soils are originally free of soybean bradyrhizobia strains which were introduced when large scale commercial crops started and the presence of *S. fredii* in soybean nodules had never been reported. Inoculation of six non-bred and one modern soybean genotypes with 22 Brazilian soil samples allowed the isolation of 30 fast-growing strains showing an acidic reaction in YMA medium. Fast growers were found in 12 of the 22 soils studied, representing 24% of total population.

The strains were characterized in relation to several phenotypic and genotypic features in order to determine the diversity of these soil populations. The preliminary phenetic analysis performed with the strains indicate that they have unique properties, but also show general characteristics of *Sinorhizobium fredii.* The dendrogram produced with several morphological and physiological parameters showed that the strains from undisturbed areas were placed into the central clusters. At the top of the dendrogram were positioned most of the strains from inoculated soils maintained under conventional tillage management, and these strains were characterized by a high tolerance to stressful conditions and the ability to grow with several C sources. At the bottom were located the majority of the strains isolated from soils managed under the non-tillage system.

The genetic clustering with ERIC and REP-PCR products showed similarities with the phenetic grouping. Though phenetic and genetic analysis indicated general similarities with *Sinorhizobium fredii* most Brazilian strains were genetically distant from three *S. fredii* type strains.

We studied the application of *nod* genes for phylogenetic studies in soybean nodulating rhizobia using some of the isolates previously studied, some *S. fredii* isolated from Chinese soils and some referential strains. These strains were examined by PCR using specific (*nodD* and *nodZ* genes) and general (*nodC* and *nifH*) genes. PCR analysis using *nod* and *nif* genes specific primers showed that Brazilian strains are genetically different from each other. They also differ from all the referential species, from *S. fredii* HH103, as well as from Chinese isolates. We have evaluated the usefulness and limitations of different primers to characterize *Sinorhizobium* strains.

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SOYBEAN BRADYRHIZOBIA STRAINS ISOLATED FROM BRAZILIAN SOILS UNDER NATIVE VEGETATION

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1. Introduction

Soybean was introduced in Brazil in 1882, but large scale commercial crop production started in the early 1960s, occupying today 13 million hectares. It is said that the soils were originally free of soybean bradyrhizobia, however, the available information is vague. The inoculants were initially brought from USA but soon a program of selection of adapted strains started. The established population found today would be then derived from previous inoculation and seeds carrying bradyrhizobia. However, in some areas inoculated two to four decades ago, a high level of diversity in relation to the putative parental inoculant strains and a high percentage of strains with unknown serological reaction have been reported. This work was then performed to verify if there is indigenous soybean bradyrhizobia in the Brazilian soils, not detected by the cultivars used in the 1960s.

2. Material and Methods

Soil samples from 14 undisturbed areas, covered with native vegetation, were collected. Each sample consisted of 20 subsamples randomly collected at a depth of 0 to 20 cm, and mixed on a sterile bag. Analyses proceeded as immediately as possible. Soybean seeds of a modern cultivar (BR-16) and six non-bred genotypes (Pickett 71, Peking, PI 245331, PI 437654, PI 88788 and PI 90763) were used to trap bradyrhizobia. Seeds were surface sterilized and inoculated with 500 µl of diluted soil (10^{-1}). At harvest, strains were isolated from nodules.

3. Results

Fourty bradyrhizobia isolates were trapped by primitive and modern genotypes and most came from the State of Amazon. Isolates were grouped into five known serogroups, of strains that had been or are now used in Brazilian inoculants, and seven isolates did not react with any of the tested antisera. For most isolates, the synthesis of indole acetic acid *in vitro* was an additional evidence for the putative parental relationship. Isolates were also grouped into eight protein and nine lipopolysaccharide profiles. The clusters obtained by ERIC-PCR were similar to those groupings based on serological reaction, protein and LPS. A high percentage of the isolates (45%) showed similar properties to SEMIA 566, a strain carried in commercial inoculants from 1966 to 1978. Consequently, the detection of this serogroup in several Brazilian sites still covered with native vegetation and far from cropping areas indicates dispersion of bacteria. Within some serogroups, the strains did not fit into the same LPS, protein and ERIC-PCR profiles. This could either indicate modifications due to the adaptation to different soil environmental conditions or symbiotic transfer to non-symbiotic native bradyrhizobia. Few isolates were very distinct from all known strains and could represent indigenous bradyrhizobia.

4. Conclusions

A high level of dispersion of soybean bradyrhizobia was verified in Brazil. It is possible that indigenous strains are also able to nodulate soybean, but they represented just a very small percentage of the population trapped with modern and non-bred soybean genotypes. Serological reaction, protein, lipopolysaccharide and DNA profiles can be modified as a result of adaptation of the strains to different soils.

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GROUPING OF RHIZOBIUM ISOLATES FROM NODULES OF COWPEA (*VIGNA UNGUICULATA*) CULTIVATED IN SOIL SAMPLES FROM THE BRAZILIAN NORTHEAST REGION BY ELISA METHOD

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Cowpea (*Vigna unguiculata*) is widely utilized by subsistence farmers who cultivate it in marginal areas without the addition of fertilizers or the use of rhizobia inoculants. Previous work performed at *Embrapa Agrobiologia* characterized morpho-physiologically a large number of isolates from nodules of cowpea cultivated in several soil samples of this region. In this study, the similarity level was evaluated among the isolates according to immunological response determined by ELISA. Cross-reactions characterized by means of the unpurified antiserum are useful to establish serological relationships among rhizobium isolates (Vincent, 1982). The results of indirect ELISA with 12 unpurified antisera against 40 isolates originated from the Brazilian northeast region were used for determining a similarity matrix worked out by a modified Bray-Curtis distance index (Ribeiro, 1999) and to cluster was utilized UPGMA method. The modification was performed in order to fulfill criteria considered relevant for this type of quantitative data. Cluster analysis revealed the formation of 10 groups at a distance of 0.5 in a full scale from 0 to 1. Six groups formed are close to *Bradyrhzobium* type strains used and they are composed mostly by isolates originated from non-irrigated areas cultivated with cowpea. Four groups were composed by isolates displaying efficient biological nitrogen fixation activity, which had little similarity with the type strains of *Bradyrhizobium* included in the analysis. The results suggest that these groups may represent a new species capable of efficiently nodulating cowpea, which may be used as an efficient inoculant for the crop. This method is relatively simple and fast and is capable of providing a reliable grouping of a large number of rhizobia isolates.

Acknowledgements

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CHARACTERIZATION OF SOYBEAN BRADYRHIZOBIA STRAINS ADAPTED TO THE BRAZILIAN SAVANNAS

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1. Introduction

The Brazilian savannas, called "Cerrados", occupy about 25% of the country's territory. Agriculture started in the region in the 1970s and soybean was introduced as the main crop. The soils were originally void of soybean bradyrhizobia and the search for strains able to establish an efficient symbiosis under the adverse conditions of Cerrados, including long dry periods, high temperatures and low soil fertility was the main approach and is used up to now. A program of selection of strains was established at Embrapa, aiming to identify isolates adapted to the Brazilian soils but with a good symbiotic performance.

2. Material and Methods

Areas of the Experimental Station of Embrapa Cerrados that two decades before showed zero nodulation and were at that time planted with soybean seeds inoculated with strain SEMIA 566 were identified. Soybean seeds were surface sterilized, planted and bacteria were reisolated from nodules showing 2-3 mm diameter and internal pink color. The strains were grouped according to the serological reactions against the antisera of all strains which had been used before in that area. The symbiotic performance of those strains belonging to serogroup SEMIA 566 was verified under greenhouse conditions and bacteria were reisolated from nodules of the treatments with higher accumulation of dry matter. Thirteen promising strains belonging to serogroup SEMIA 566 were obtained using this approach: S-127, S-204, S-340, S-370, S-372, S-406, S-452, S-468, S-478, S-481, S-490, S-506 and S-516.

3. Results

All variant strains belonging to serogroup SEMIA 566 showed similar profiles of lipopolysaccharides and proteins to those observed for the parental strain SEMIA 566. The variant strains also produced more mucus and lower amounts of indole acetic acid *in vitro* than SEMIA 566. The DNA profiles obtained by the amplification with arbitrary and short primers (RAPD) and ERIC and REP-PCR confirmed a relationship between the parental and variant strains. However, DNA polymorphisms were detected in the adapted strains, and could result from the process of adaptation to the inhospitable environmental conditions of Cerrados. Differences in symbiotic performance were also detected, with five of the 13 variant strains accumulating more N in tissues than SEMIA 566. Although SEMIA 566 is a very competitive strain, two of the variant strains were as competitive as SEMIA 566 and the other 11 variant strains were even more competitive than the parental genotype.

4. Conclusions

Adaptation to stressful environmental conditions causes morphological, physiological and genetic changes in soybean bradyrhizobia. However, within this genetic variability it is possible to select efficient, competitive and stress tolerant strains able to increase the contribution of N_2 fixation to the soybean nutrition.

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GENOTYPIC DIVERSITY OF *BRADYRHIZOBIUM* STRAINS OF TROPICAL AND TEMPERATE ORIGIN AND THE IDENTIFICATION OF A NEW GENOMIC SPECIES NODULATING ENDEMIC WOODY LEGUMES (FABACEAE:GENISTEAE) FROM THE CANARY ISLANDS

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We have genotypically characterized a collection of diverse *Bradyrhizobium* strains of tropical and temperate origin by means of 16S-, 23S- and IGS-rDNA PCR/RFLPs, partial and full-length 16S rDNA sequencing, rep-PCR genomic fingerprinting using REP and BOX primers, genomic fingerprinting using the *B. japonicum* USDA110 RSα and RSβ IS-like repetitive sequences as hybridization probes, and DNA-DNA hybridization analysis with selected strains. The strains were isolated from 25 different hosts (belonging to 10 tribes) and 22 countries in 5 continents. Besides well-characterized reference strains of *B. elkanii, B. japonicum* and *Bradyrhizobium* sp., over 100 new isolates included in our open relational database.

We determined the genetic affinities of nodule isolates from several genera of endemic woody legumes (Papilionoideae:Genisteae) of the Canary Islands, including plants of notable ecological and agronomic interest such as *Adenocarpm* spp., *Chamaecytisus proliferus, Spartocytisus* spp. and *Teline* spp. This study extends previously published work on partial phenotypic (León-Barrios et al., 1991; Santamaría et al., 1997) and genotypic (Vinuesa et al., 1998, 1999) characterization of a small subset of these Canarian strains. We found that PCR/RFLP analysis of the nearly full-length *rrn* operon provides much greater taxonomic resolution than full-length 16S rRNA gene sequencing, allowing the identification of novel genotypes within strains belonging to the taxonomically difficult *B. japonicum/R. palustris* 16S rDNA phylogenetic branch. For example, we found several new *Bradyrhizobium* lineages that are related neither to the *B. japonicum* nor to the *B. elkanii* rDNA branches. The photosynthetic isolates from *Aeschynomene* spp. form a separate cluster that is more related to the *B. japonicum* than to the *B. elkanii* rDNA branch. A further distinctive and well-resolved cluster contains some of the Canarian strains. This ”Canarian *Bradyrhizobium* cluster” is the only one for which a strict correlation between geographic origin and rDNA genotype was found. DNA-DNA hybridization data indicate that the strains in this cluster represent a new genomic species related to the *B. japonicum* 16S rDNA lineage, as inferred from our rDNA PCR/RFLP analysis and confirmed by full-length 16S rRNA gene sequence analysis. However, a detailed phenotypic characterization of these novel genotypes is required before formal decissions of their taxonomic status. This phenotypic characterization is currently being performed for the strains in the novel Canarian *Bradyrhizobium* rDNA cluster.

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DIVERSIFICATION OF SOME FORAGE LEGUMES RHIZOBIA ISOLATED IN THAILAND

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Atmospheric N_2 was able to be biologically fixed through forage legumes and provided not only enough N for their growth but also released into soil. Therefore, rhizobia varied in their effectiveness in fixing N_2 to be more benefit from N_2 fixation, it is important to select high effective strains of forage legume rhizobia to inoculate seeds before planting. In this study effective rhizobia strains in four tropical forage legumes were selected according to their N_2-fixing efficiency and characterized in both terms genotypic and phenotypic. Soil samples from North and Northeast of Thailand were collected for isolating rhizobia by using forage legumes as trapping host plants. *Desmanthus virgatus, Stylasanthes hamata, Chamaecrista rotundifolia, Centrosema pubescens* and *Centrocema pascuorum* (cavalcade) were choosen as host plants for isolation of rhizobial strains from their nodules. The high efficiency of N_2 fixing strains were deteced by ARA assays. In order to investigate their physiological properties, colour reaction on YMA + BTB medium, IAA production, antibiotic resistance profiles and 19 different substrates utilization (APIXYM-test) were achieved, while to determine their genomic fingerprints, *nif* and *nod* genes were employed and RAPD-, REP-PCR for distinguishing the strains also be conducted. Characterization of 24 strains of *D. virgatus* rhizobial strains, the results indicated that most of them were fast-grower group while other plant hosts rhizobia comprised of both fast-grower and slow-grower. In addition by using antibiotic resistant it was cleary that most of strains were susceptible to erythromycin. Moreover, among these strains also found that neither α-monosidase nor α-fucosidase were produced from these strains. On the other hand, several enzymes involved in carbohydrate degradation were found such as α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase and N-acetyl-glucosamidase. Moreover, some *S. hamata* rhizobial strains exhibited the enzyme activities profiles with the same patterns as most bradyrhizobial strains. In case of *D. virgatus* rhizobia, one of them seemed to be the *Rhizobium tropici* and the rest were closely related in intraspecies level. This was confirmed by cross nodulation between rhizobial strains and plant hosts such as *Phaseolus vulgaris* and *D. virgatus* prior to detected with direct-nodule PCR approach. However, when distinguish the other plant hosts rhizobia by using random primers the results suggested that in each plant host, great diversity of rhizobia were found.

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AZORHIZOBIUM JOHANNENSE SP. NOV. AND *SESBANIA VIRGATA* (CAZ.) PERS.: A HIGHLY SPECIFIC SYMBIOSIS

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Thirty one fast and intermediary growing rhizobia strains with cultural characteristics on YMA similar to *Azorhizobium caulinodans* were isolated predominantly from the fast grow native species *Sesbania virgata* at different regions at South East Brazil (Minas Gerais and Rio de Janeiro). SDS-Polyacrylamide gel electrophoresis of total bacterial proteins were performed by small modifications of the procedure of Laemmli (1970) as described previously (Kiredjan *et al.,* 1986). All strains have relatively similar total protein profiles and presented small differences as compared to *Azorhizobium caulinodans* ORS 571. Growth and nitrogenase activity under free living conditions were tested in semi-solid and solid N–free LO modified medium (Dreyfus, 1982). Nitrogenase activity was estimated by using acetylene reduction assay. Ethylene production was detected with flame ionisation gas chromatograph. In LO solid medium with bromothimol blue all strains grew alkalinizing the medium and absorbing the indicator, thus having a blue colour. In semi solid LO modified medium all strains of *Azorhizobium* sp. nov. grew forming a pellicle near surface just like ORS 571, however, their pellicles have a slower growth, i. e, they reach surface after the pellicle of ORS 571 and nitrogenase activity (ARA) was smaller than that of ORS 571. All strains and ORS 571 grew on LB medium and do not acidified glucose medium, instead there was a strong alkali production. Plant infection was tested with *S. virgata* and *S. rostrata* growing in Leonard jars (Vincent, 1970), containing a mixture of sand and vermiculite (1:1) with Jensen's modified nutrient solution. Each treatment was carried out with 3 jars (2 plants /jar). Species were analysed for: plant dry weight, nodule numbers, shape and dry weight and means were compared by Tuckey' s test. Both *A. caulinodans* and *A.* sp. nov. strains are able to nodulate roots of *S. rostrata* and *S. virgata.* However *A.* sp. nov. nodules are inefficient in *S. rostrata* and *A. caulinodans* nodulates efficiently *S. virgata.* The thirty one isolates of *Azorhizobium* sp. nov. exhibited a high similarity of phenotypic characteristics among them and to a lesser extent to *A. caulinodans,* which is the most similar known rhizobia species to them. Partial 16S rRNA sequence strongly support this affinity as the most similar published sequence to Br 5401 is ORS 571 (Moreira et al., 1998). However, the eight different bases also support its dissimilarity with *A. caulinodans* which is in accordance with other phenotypic and symbiotic differences found such as protein profiles, nitrogenase activity, lack of stem nodulation. This species cannot be confound with another two distinct species found nodulating *S. rostrata* by Rinaudo st al. (1991). One of those did not fix nitrogen in free-living conditions and the other does nodulate *S. rostrata* stems which are not characteristics *of Azorhizobium* sp. nov. On the basis of their peculiar characteristics and eight different bases in the partial 16S rDNA sequence, it is proposed as a new species in genus *Azorhizobium.*

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DIAZOTROPHS THAT GROUP WITHIN THE PSEUDOMONADACEAE BASED ON PHYLOGENETIC EVIDENCE

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1. Introduction

The lack of ability to fix nitrogen has been considered to be a characteristic of the family Pseudomonadaceae, yet there is evidence that supports the existence of diazotrophic *Pseudomonas* species (Krotzky,Werner, 1987). This study represents an effort to determine phylogenetic relationships between *Pseudomonas,* diazotrophic organisms recently isolated from natural environments (Loveless et al., 1999) and members of the family Azotobacteriaceae.

2. Materials and Methods

Aligned concatenated sequences of small subunit (ssu) ribosomal RNA and RNase P RNA genes were used as the basis for this phylogenetic study. Evolutionary distance was estimated by the Jukes-Cantor method using DNADIST (Felsenstein, 1993). A tree was constructed from the concatenated RNase P RNA: ssu rRNA alignment using the least squares, distance matrix-based program DNAML (Felsenstein, 1993). A tree constructed using DNA parsimony (DNAPARS; Felsenstein, 1993) yielded a similar tree except for some of the fine-scale branching orders.

3. Results

The phylogenetic trees consistently placed the diazotrophs interspersed with the two *Pseudomonas* species, *P. aeruginosa* and *P. fluorescens.* Sequence identity and evolutionary distance from the RNase P RNA and ssu rRNA gene sequences is as similar between the *Pseudomonas* species as it is between these species and the diazotrophic organisms. Thus, the genus *Pseudomonas,* if it is to remain a monophyletic taxonomic group, should contain the nitrogen-fixing environmental isolates, and the members of the Azotobacteriaceae (*A. vinelandii, A. paspali, A. salinestris,* and *Azomonas macrocytogenes*).

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GENOTYPIC, PHENOTYPIC AND SYMBIOTIC DIVERSITY AMONGST RHIZOBIA ISOLATES FROM *PHASEOLUS VULGARIS* L. GROWING IN THE AMAZON REGION

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Phaseolus vulgaris L. is a highly promiscuous host that can be nodulated by at least 8 rhizobia species: *Rhizobium leguminosarum* bv phaseoli, *Mesorhizobium loti, Sinorhizobium fredii, M. huakuii, R. tropici, R. etli, R. giardinii* and *R. gallicum.* Main constraints to biological nitrogen fixation (BNF) in beans relating to rhizobia are: the genetic instability (e.g. *R. leguminosarum* and *R. etli*), low competitiveness of inoculant strains with the indigenous population, usually considered inefficient, and sensitivity to environmental factors such as soil acidity and high temperatures. *R. tropici* is being considered the most promising species to select inoculant strains because its reasonably genetic stability, tolerance to acidity and to high temperature and widespread occurrence in tropical regions. Despite of that, in many experiments carried out in Brazil, no response to inoculation with *R. tropici* CIAT 899 has been found. Although, *R. tropici* description has been based on a majority of isolates from Brazilian soils (mainly from "Cerrado"), there is no data showing its predominance in those soils. Recently, Mercante et al. (1998) found that only 36% of beans isolates from "Cerrado" soils could be classified as *R. tropici.* The objective of this study was the phenotypic, genotypic and symbiotic characterisation of indigenous rhizobia population nodulating *Phaseolus vulgaris* L. Forty four rhizobia strains were isolated from nodules of non-inoculated *Phaseolus vulgaris* cv. Carioca, collected in two recently deforested areas of Theobroma Municipality, Rondônia state, in may 1997. Strains UFLA 49 to 72 were isolated from area 1 and UFLA 84 to 139 from area 2. All strains and type strains of *Rhizobium leguminosarum* bv. *phaseoli, R. tropici, R. etli* and *Sinorhizobium fredii* (from CNPAB-EMBRAPA, Rio de Janeiro) were analysed for: cultural characteristics on YMA, total protein profiles by SDS- Polyacrylamide gel electrophoresis, 16S ribossome DNA (rDNA) PCR - Restriction length polymorphism (Hinf, RSA, Alu, Hae) and symbiotic properties with *Phaseolus vulgaris* cv. Carioca cultivated both on plastic pouches and Leonard jars with nutrient solution. All 44 isolates were able to induce nodules in *Phaseolus vulgaris* cultivated in plastic pouches with nutrient solution. Strains from both areas exhibited a high heterogeneity both by total protein SDS-PAGE profiles and PCR-RFLP of 16 s rDNA. Nine eletrophoretic protein groups were obtained: six from area 1 and five from area 2, being two of them (GI and GII) common to both areas. Strains belonging to each group are: GI [UFLA (49, 52, 55, 70, 72, 108, 115, 122, 127 and 138)]; GII [UFLA (61, 84, 85, 86, 87, 90, 92, 97, 100, 102, 103, 104, 105, 106, 110, 116, 117, 132, 133, 134)]; GIII [UFLA (54, 60, 65, 66)]; GIV [UFLA (67, 68)]; GV (UFLA 91); GVI (UFLA 113); GVII (UFLA 63); GVIII (UFLA 64) and GIX (UFLA 139). A highest similarity of all of these groups was found with *R. leguminosarum/ R. etli* type strains. Majority of strains belonging to different SDS-PAGE groups, presented similar restriction patterns. Exceptions were strains 60, 139, 85, 110, 113 and 127. The most discriminating restriction enzyme was Hae. Fifteen strains presented different efficiencies in promote plant growth. All of them except UFLA 60 increase growth in relation to the control. Plant growth with the most efficient strain (UFLA 127) corresponded to 70% plant dry weight as compared with those receiving 140 mg L^{-1} N-NH_4NO_3 and 165% as compared of those inoculated with Br 322/CIAT 899. No relation was found amongst efficiency and SDS-PAGE group.

DIVERSITY OF *BRADYRHIZOBIUM* STRAINS ISOLATED FROM ENDEMIC WOODY LEGUMES OF THE CANARY ISLANDS BY PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF 16S RDNA AND LOW-MOLECULAR-WEIGHT RNA PROFILES

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The *Bradyrhizobium* species currently admitted are three (*B. japonicum, B. elkanii* and *B. liaoningense*), but this number is expected to increase with the analysis of more strains isolated from non-previously studied legumes. In the Canary Islands a large number of endemic shrub legumes of great agronomic interest grow. Tagasaste (*Chamaecytisus proliferus*) has specially been used for centuries as fodder on the Canaries and other parts of the world. In previous works with some rhizobia isolates from tagasaste and other endemic woody legumes, it was shown that these legumes were nodulated by a group of *Bradyrhizobium* strains phenotypically and genetically diverse (León-Barrios et al., 1991; Santamaría et al., 1997; Vinuesa et al., 1998). In this study, thirty-six strains of slow-growing rhizobia isolated from endemic woody legumes were characterized by analysis of PCR-amplified and restricted 16S rDNA, and by LMW RNA profiles. The isolates, incapable of nodulating soybean, were compared with reference strains of genus *Bradyrhizobium.* Both techniques showed similar results, clustering the isolates in two main groups. The PCR-amplified 16S rDNA was individually restricted with four endonucleases, and the combined RFLP patterns used for cluster analysis by UPGMA. This analysis showed a larger group (29 strains) containing only Canarian isolates (BTA-1 group), and two strains of *Lupinus.* One smaller group of nine strains (BGA-1 group) clustered with *B. japonicum* strains. None of the isolates grouped with the strains of *B. elkanii.* RNA profiles in the 5S rRNA zone showed a pattern identical to *B. japonicum* for both groups. However, tRNA profiles of both groups were different from that of *B. japonicum,* although BGA-1 group profiles are more similar to this species. According to previous results (Velázquez et al., 1998), the different species present different tRNA profiles. Thus, the results obtained from both techniques suggest that some Canarian isolates appear to be new species of genus *Bradyrhizobium.* This idea is also supported by partial sequencing of 16S rRNA gene (Vinuesa et al., 1998). However, new data will be needed to confirm it, especially total DNA hydridization. The finding of two strains of *Bradyrhizobium* sp. (*Lupinus*) clustering at a similar level of 100% with the BTA-1 group by ARDRA analysis was a surprise. It had been found (Vinuesa, 1998) that RFLP analysis of rDNA operon could separate a group of ten Canarian isolates among a vast collection of bradyrhizobia, isolates from different parts of the world, giving strong evidence that they could represent a local population of bradyrhizobia confined to the Canary Islands. However, the fact of two strains of *Lupinus* collected in geographically unrelated locations (Sevilla and Madrid, Spain-mainland), that can nodulate tagasaste and clustering in the same ARDRA group as the "Canarian group", means that more investigation should be carried out to clear up this matter. Data reflecting total genome variation will be very interesting.

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MOLECULAR CHARACTERIZATION OF NEW DIAZOTROPH GENOTYPES FROM *MUSA* SPP. AND *ANANAS* SPP.

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For decades several nitrogen-fixing bacteria have been isolated from graminaceous plants and reported to benefit their hosts but, recently their range has been extended to different plant species of economic importance, such as coffee and oil palm (Baldani et al., 1997). Weber et al. (1999) also showed the association of diazotrophic organisms with banana and pineapple. According to physiological analysis and hybridization with species-specific oligonucleotide probes these authors showed a relationship between the isolates and species of *Azospirillum, Herbaspirillum,* and *Burkholderia.*

In the present work, molecular techniques were employed to characterize 38 pineapple and banana isolates and 5 reference strains from *Herbaspirillum seropedicae* (strains Z67 and M2), *Herbaspirillum rubrisubalbicans* (strain M4), *Burkholderia brasilensis* (strain M130), and *Burkholderia tropicalis* (strain Ppe8). Primarily, the 16S – 23 S rDNA intergenic region and 16S rDNA were separately amplified by polymerase chain reaction (PCR). The 16S – 23 S rDNA intergenic region-PCR products were digested with *Alu*I, *Hae*III, and *Rsa*I restriction endonucleases and the fragments separated in a 2.0% agarose gel electrophoresis. This analysis clustered the isolates in 13 genotypes. Similarly, the 16S rDNA-PCR products were digested with the restriction endonucleases *Alu*I, *Hae*III, *Hinf*I, and *Rsa*I and the fragments separated undo the conditions described above, in a so-called amplified 16S ribosomal DNA restriction analysis (ARDRA). Restriction fragment patterns analyses indicated the existence of 12 different genotypes, confirming the previous analysis. It also identified 16 isolates sharing ARDRA patterns identical to the four reference species. However, 22 strains belonging to 8 ARDRA genotypes were found to be distinct from the reference strains. Finally, the 16S rDNA-PCR products were also used to sequence approximately 300 bp in the 5′ end region of the molecule. This is a variable region of the 16S rDNA, which is useful for phylogenetic comparison of close relatives (Young et al., 1991). Analysis showed that 3 unidentified ARDRA genotypes belonged to *Azospirillum,* 4 to *Burkholderia* and 1 was located separately within the beta subclass of the *Proteobacteria,* clustered with Comamonadacea species. The isolates classified as *H. seropedicae*, *B. brasilensis*, and *B. tropicalis* by ARDRA were confirmed, showing 100% identity with the reference strains. However, isolates clustered with the *H. rubrisubalbicans* reference strain by ARDRA were ambiguous, showing 100% identity with *H. seropedicae* in the sequenced region. The *Herbaspirillum* genotypes were particularly difficult to differentiate due to their close relationship. They showed no difference in the restriction patterns of the 16S – 23 S rDNA intergenic regions, a single different band only in the *Alu*I restriction pattern for ARDRA, and only two base differences in the sequenced region. Weber et al. (1999) also found ambiguity in the physiological and hybridization tests for the same isolates.

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CHARACTERIZATION OF *SINORHIZOBIUM FREDII* STRAINS ISOLATED FROM CHINA SOILS

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Soybean (*Glycine max* (L.) Merrill) can be nodulated by slow-growing bacteria (*B. japonicum*) and by fast-growing bacteria (*S. fredii* or *S. xinjiangensis*). Traditionally, *B. japonicum* has been used for inoculant production, but in the last decade, different groups have reported the isolation of *Sinorhizobium* strains that are able to nodulate and form effective **N_2-fixing** symbiosis with American soybean cultivars (Dowdle and Bohool, 1985; Balatti and Pueppke, 1992; Buendía-Clavería et al., 1994). A total of thirty-three fast-growing isolates were sampled in Hohghu county (province of Hubei) using as trap-plant an American soybean cultivar (Williams) as well as an Asiatic one (Peking). They were characterized by eighteen physiological traits, plasmid, proteins and LPS profiles and ERIC/REP-PCR products. Reference type strains of different *Rhizobium* species were included. A dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic means (UPGMA) using the physiological traits and the PCR fingerpritings.

This dendrogram allowed us to determine the genetic relationships among *Sinorhizobium* isolates of this work and the reference strains used. The cluster I contains 31 strains and could fit into the description of *S. fredii* species. It is divided in two subclusters: subcluster I-1 includes 14 Chinese isolates and the reference strains of *S. fredii* (USDA 205 and HH 103), that might correspond with *S. fredii* cv. *fredii.* Subcluster I-2 includes 15 Chinese strains, which raised a final pH on YEM broth higher than 6.0, so they could fit into the chemovar *sinensis.* Cluster II is formed by three Chinese strains and the reference strain CC BAU 110, so they could belong to *S. xingiangensis* species. Strain CH 16 was closely related to *R. tropici* CIAT 899 and *R. meliloti* RCR 2011 (cluster III). This strain should be considered as unique until more date are available.

According to the different techniques used in this work, it is clear that the physiological parameters as well as the PCR fingerprinting are useful to obtain information about the genetic relationships among the isolates and to achive a preliminar classification. However, we could not see differences between some isolates, which means that these techniques can not be succesfully used for rhizobial strains differentiation. In contrast, plasmid profiling analysis was more useful than other techniques applied in terms of discriminating ability among strains.

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DIVERSITY IN THE MCROSYMBIONT POPULATION ASSOCIATED WITH WILD BEANS FROM THE NORTH WEST OF ARGENTINA.

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The Northwest of Argentina (NWA) is considered to be one of the two main areas of bean domestication. Wild bean *Phaseolus vulgaris* var. aborigineus shares its habitat with others wild legumes such as *Phaseolus augusti, Crotalaria, Desmodium,* and *Acacia aroma.*
Single legume species are often nodulated by different bacterial species or genera and most rhizobia can nodulate several legumes species and genera. *Leucaena leucocephala* is a permissive host that can be used for trapping from soils, a broad range of genotypes, including those that are also able to nodulate beans.

We report here the results of analysis of 100 rhizobial isolates from nodules *L. leucocephala* plants, which had been inoculated with soils suspensions from different geographic locations, mainly from Salta province. Similarly, a population isolated from nodules of the perennial wild bean *Phaseolus augusti* collected in the Andean slopes was analysed. Genetic diversity was assessed by 16S rDNA RFLP, intergenic region (IGS) 16S-23S rDNA RFLP, DNA fingerprints, plasmid profiles.

We found eight 16S rDNA (Weisburg et al., 1991) genotypes isolated from *L leucocephala,* some of them were found similar to alleles of *S. fredii, S. saheli, S. teranga* and *R. tropici* (de Lajudie et al., 1994). RFLP analysis of the IGS (Laguerre et al., 1996) region of the isolates belonging to each 16S rRNA allele were found to be diverse. The isolates from *P. augusti* generated only two 16S rRNA genotypes that were found similar to *B. elkanii* and *B. japonicum.* By IGS, isolates *B. elkanii*-like were clustered together. Genomic fingerprints using ERIC- and REP-PCR (Versalovic et al., 1994) resulted in a diversity of patterns with fragment sizes ranging from 0.2-2.5 kb. Twenty seven representative isolates from *P. augusti* generated twenty different patterns of RFLP of PCR-amplified *nifli* gene, which indicates a high variability in the symbiotic gene *nifH.* Contrarily, isolates from *L. leucocephala* with a *S.fredii* 16S rRNA allele, were clustered together.

Isolates from leucaena having different 16S rRNA alleles were found to form nodules on common beans under laboratory conditions. However we were unable to find them associated to beans growing in NWA. The slow grower rhizobia isolated from *P. augusti* induced small and white nodules in *P. vulgaris.*

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THE NODA SEQUENCE OF RHIZOBIA GIVES CLUES ON STRUCTURAL FEATURES OF NOD FACTORS

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Lipo-chitooligosaccharide Nod factors (NFs) synthesized by rhizobia act as signal molecules in the nodulation of specific legume hosts. NFs generally consist of four or five glucosamine residues that are N-acylated at the non reducing end and carry other substitutions on various glucosamine residues. Each rhizobial species (or biovar) has a defined host range and produces a set of NFs with specific structural features involved in host range determination.

The determination of NF structures represents heavy work and is only suitable for a limited number of strains. Taking advantage of the recent development of DNA molecular techniques, we attempted to find out whether the analysis of the *nodA* gene sequence could help us predict NF structures. The *nodA* gene is required for NF synthesis and is present in a single copy in all rhizobia. The NodA protein is involved in the specific transfer of an acyl chain to the chito-oligosaccharide backbone of NFs (Debellé et al., 1996; Ritsema et al., 1996; Quinto et al., 1997; Schultze et al., 1995). NodA thus interacts with two substrates, both varying in structure among different rhizobia. We focused on the *Sinorhizobium-Mesorhizobium-Allorhizobium-Rhizobium* branch since this branch represents maximum NF and taxonomical diversity.

We sequenced the entire *nodA* gene of about 20 strains whose NF have been structurally identified, by using several primers designed by comparing *nodA, nodB* and *nod* box sequences available in GeneBank database. A phylogenetic tree was constructed using all the available NodA sequences of the *Sinorhizobium-Mesorhizobium-Allorhizobium-Rhizobium* branch. The NodA proteins formed several clusters which do not correlate with the taxonomical position of the rhizobial strains, but rather with structural features of NFs such as fucosylation, arabinosylation and the presence of α,β-unsaturated fatty acids. We thus classified NFs into four types, G, F, T and A, depending on the structure of their acyl chain and the presence and structure of additional glycosyl groups. Having a set of aligned NodA sequences of known NF types, the probability of NF types (G, F, T or A) can be estimated for a given NodA sequence, and the NF type can be predicted by the type which shows the maximum probability. To validate the prediction of the NF type, we used the Jacknife procedure, i.e. each sequence was removed from the set of aligned sequence, its NF type was predicted and compared with the known NF type.

These results indicate that the NodA sequence of a strain may help predict the type of the produced NF. As a consequence, i) *nodA* sequencing may be used as a tool to search for novel NF structures; ii) *nodA* sequencing may be used to predict the ancestral NF structure and its evolution in the course of legume evolution.

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PHYLOGENETIC COMPARISON OF SYMBIOTIC (*NODC* AND *NIFH*) AND 16S rRNA GENES IN STRAINS OF *RHIZOBIUM, MESORHIZOBIUM* AND *BRADYRHIZOBIUM* ISOLATED FROM *ASTRAGALUS, OXYTROPIS* AND *ONOBRYCHIS* SPP.

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Various studies reported different conclusions on the phylogenetic relationship between symbiotic *(nod* and *nif)* and chromosomal (16S rRNA) genes in rhizobia (Haukka et al, 1998). Some studies suggested that the phylogeny of *nod* genes could be related with the host plant (Ueda et al., 1995). In a previous study, rhizobia (44 isolates) from *Astragalus*, *Oxytropis* and *Onobrychis pp* have been classified into 14 distinct 16S rRNA gene types independently of their host plant and geographic origin. The majority of isolates were classified within the genus *Mesorhizobium,* and few within *Rhizobium* and *Bradyrhizobium* genera (Laguerre et al., 1997). The aim of the present study was to determine the diversity and phylogeny of symbiotic genes (*nodC* and *nifH*) of these rhizobia, to compare the distribution of *nodC* and *nifH* types to that of 16S rDNA types, and to evaluate the relation between host nodulation range and phylogeny.

Variation in symbiotic genes was estimated by PCR-RFLP analysis of *nodC* gene regions using two endonucleases (*MspI, HinfI*) and *nifH* gene regions using four more enzymes (*AluI, CfoI, HaeIII, NdeII). NodC* genes of different *nodC* types were sequenced and a phylogenetic tree was constructed. Host nodulation range was determined by inoculating two to six plants of each plant species (11 plant species) with each strain. Nodulation was recorded positive after reisolation from nodules.

The number of *nodC* and *nifH* gene types was sligthly higher than that of 16S rDNA types, suggesting a greater diversity in symbiotic genes than in highly conserved ribosomal genes. Within 16S rDNA types and the three genera, the *nodC* types were similar among isolates but differed from those of the type strains of the corresponding rhizobial species, except for one isolate grouped with *R. leguminosarum* bv. viciae. The grouping of isolates in the phylogenetic tree based on *nodC* sequences ressembled to that in the tree based on 16S rRNA genes. *NifH* types were similar within 16S rDNA types, but showed more variations within the genus *Mesorhizobium.* The phylogenetic congruence between symbiotic genes and 16S rRNA genes at the genus level suggests that Sym gene transfer does not occur across genera. Most isolates could nodulate plant genera of origin (*Astragalus*, *Oxytropis* and *Onobrychis*) which are phylogenetically related. None or very few isolates grouped with reference strains of *M. loti* or *M.ciceri* could nodulate *L .corniculatus* or *C. arietinum.* Isolates of *R.gallicum* could nodulate *P. vulgaris,* but this legume was also nodulated by many isolates from all 16S rDNA types. The legumes *M. saliva* and *G. max* were nodulated (non-efficiently) by isolates from the three rhizobial genera. The non relationship between phylogeny (symbiotic and ribosomal genes) with the host nodulation range may suggest a specific evolution pattern in rhizobia of our study.

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IDENTIFICATION OF PUTATIVE *NIF*DK GENES IN THE GENOME OF A. *BURKHOLDERIA* LIVING IN SYMBIOSIS WITH AN ARBUSCULAR MYCORRHIZAL FUNGUS

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1. Introduction and Results

The widespread association between soil-born fungi and plant roots is known as mycorrhizal symbiosis. An arbuscular mycorrhizal fungus (*Gigaspora margarita* BEG 34) has been demonstrated to harbour a homogeneous population of endosymbiont *Burkholderia* (Bianciotto et al., 1996). A genomic library of total DNA extracted from the fungal spores was also representative of the bacterial genome, and was used to investigate the prokaryotic genome (van Buuren et al., 1999). The aim of the work was to demonstrate that a DNA region contains putative *nif* genes belonging to the *Burkholderia.* Screening of the library with *Azospirillum brasilense nifDK* genes as the prokaryotic probe led to the identification of a 3380 bp region. The analysis of the nucleotide sequence revealed the presence of two complete open reading frames encoding putative proteins exhibiting a very high degree of sequence similarity to the two subunits (NifD and NifK) of the component I of nitrogenase from different diazotrophs. The two genes were arranged in an operon similar to that shown by most archaeal and bacterial diazotrophs. PCR experiments with primers specifically designed on the *Burkholderia nifDK* genes and Southern blot analysis demonstrate that they actually belong to the genome of the *G. margarita* endosymbiont. A phylogenetic analysis performed on the available *nifK* sequences, including that obtained from *Burkholderia vietnamiensis* TVV75, which is known to fix N_2, placed the endosymbiotic *Burkholderia* closer to *Azospirillum brasilense* than to free-living *B. vietnamiensis* TVV75. This is the first demonstration that an AM fungus contains symbiotic intracellular bacteria possessing *nif* genes in their genome. The significance of this finding (the potential capacity of a mycorrhizal fungus to fix nitrogen through a specific bacterial population) lies in the interest of such a combination for sustainable agriculture. The natural system consisting of mycorrhizal fungi and nitrogen-fixing bacteria may be an excellent "biofertiliser" with which to expand crop production and minimise the negative impact of chemical fertilisers.

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RHIZOBIAL POPULATIONS NODULATING *LEUCAENA LEUCOCEPHALA, MIMOSA AFFINIS,* AND *SESBANIA HERBACEA* IN MEXICAN SOILS

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Up-to-date, 27 species forming nitrogen-fixing root-and/or stem-nodules on legumes within 6 genera have been described for these bacteria. It is believed that the original center of a host legume is also the center where the rhizobia associated to it diverges. *Leucaena leucocephala, Mimosa affinis*, and *Sesbania herbacea* are legumes are native to Mexico.Rhizobial populations consisting of 160 isolates from the 3 host species and some reference strains for defined rhizobial species and unnamed lineage were characterized by restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rRNA genes and to estimate their phylogenetic relationships. Multilocus enzyme electrophoresis (MLEE) analysis was performed to assay the genetic diversity for different populations according to the host plants or the geographic origins. Forty-one electrophoretic types (ETs) were identified among 150 isolates from *L. leucocephala* and they were clusters into groups in general agreement with the 18 rDNA types corresponding to *Mesorhizobium, Rhizobium,* and *Sinorhizobium* based on PCR-based RFLP of 16S rRNA genes. Sixteen ETs were identified among 50 isolates fromM *ajfinis* growing in different regions of Mexico. They were clusters into two groups corresponding to *R. etli* and a novel group (rDNA type) within the genus *Rhizobium* , in which isolates fromM *affinis* and *L. leucocephala* were intermingled. Four rDNA types corresponding to *Rhizobium huautlense, R. tropici* type B, *Rhizobium* sp. and *Mesorhizobium plurifarium* were identified among the isolates from *S. herbacea.* The *Mesorhizobium* isolates formed a single cluster with those from *L leucocephala* in MLEE analysis and was identified as *M. plurifarium* based on DNA hibridization and phenotypic characteristics. Diverse plasmid patterns were obtained among the isolates. The symbiotic plasmids were identified among most of the isolates, except for the *Mesorhizobium* isolates from *L. leucocephala* and *S. herbacea* and among the others isolates from *L. leucocephala.* Multiple *nifH* copies were found among the fast growing rhizobia associated with *S. herbacea* and *M. affinis.*

Conclusions

1.-The rhizobial populations associated with the 3 legume species tested were very divergent. 2.-Some of the rhizobial groups were common for the 3 legume species and others are unique for each of the hosts. 3.-Nodulation on *S. herbacea* in flooded soils is a unique feature for *R. huautlense* and flooding is a factor affecting rhizobial diversity associate with this plant.

SECTION IV

LEGUME SYMBIOSIS

CONTROL OF NODULATION IN LEGUME SYMBIOSIS

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The intimate interaction between rhizobia and their legume hosts is known to involve the mutual exchange of diffusible signal molecules. Although past research has largely focused on the plant flavonoid signals that induce bacterial *nod* gene expression and the return bacterial Nod signal that induces nodule formation, it is now clear that a multitude of additional signals are likely involved in the nodulation process. These additional signals include bacterial surface polysaccharides and excreted proteins. Additional plant signals are also recognized by the rhizobial symbiont. Therefore, although the lipo-chitin Nod signals are the most important for nodulation, their role must be viewed against the backdrop of the extensive exchange of signals between host and symbiont.

Rhizobial Nod signals are substituted lipo-chitin oligomers (LCO), usually of four to five *N*-acetylglucosamine (GlcNAc) residues, mono-N-acylated at the non-reducing end and carrying a variety of substitutions at both the reducing and non-reducing terminal GlcNAc residues. Each rhizobial species produces a variety of Nod signals with specific substitutions (Denarie et al. 1996). Inoculation of roots with the appropriate Nod signal will elicit many of the responses seen upon rhizobial inoculation (reviewed in Conn et al., 1998). The fact that these LCO signals act at very low concentrations and exhibit chemical specificity suggests that perception is likely mediated by specific protein receptors. Therefore, it is not surprising that a considerable amount of effort is focused on identifying these receptors.

A variety of approaches are being used in various laboratories to identify the Nod signal receptor and to elucidate the down stream signaling events. For example, several groups are utilizing map based (positional) cloning methods to isolate genes, mutations in which disrupt early nodulation events. The hope is that one or more of these genes may encode the Nod signal receptor. Although these efforts have not yet borne fruit, it can be anticipated that many important genes involved in Nod signal perception will be identified through this approach.

The group in Toulouse, France, represented at the meeting by Dr. Julie Cullimore, is one of the leading groups seeking to identify the receptor(s) for the Nod signal produced by *Sinorhizobium meliloti.* This group has been using a biochemical approach using radioactively labeled LCO to identify binding sites on plasma membranes isolated from alfalfa Two binding sites have been identified and one, NFBS2, exhibited a high affinity (Kd= 2 nM) for the *S. meliloti* Nod signal. Recently, this binding site was solubilized from plasma membrane preparations from *M. varia.* The solubilized protein showed specific binding to the *S. meliloti* Nod signal and could discriminate based on fatty acid chain length, the degree of polymerization of the oligochitin backbone, and the presence of an O-acetyl group at the non-reducing end of the molecule. However, binding to NFBS2 was not dependent on sulfation of the LCO, which is a critical determinant for biological activity on alfalfa. This latter result leaves in question the exact role NFBS2 plays in nodulation of *Medicago.* However, this binding site still represents perhaps the best candidate for a bonafide Nod signal receptor.

In soybean, simple chitin oligomers have been shown to activate the expression of the early nodulin, ENOD40 (Minami et al., 1996). These data led us to propose the hypothesis that the Nod signal

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receptor could be a member of a general class of chitin-binding proteins in plants. Using a similar approach to that of the Toulouse group, we have identified a chitin binding protein in the plasma membrane. The data suggest that this protein may play a role in activating a defense response (e.g., oxidative burst) in response to fungal cell wall chitin fragments.

A final approach to identify a Nod signal receptor is that reported by Etzler et al. (1996). They identified a lectin, DB46, from the roots of the legume *Dolichos biflorus,* that could bind LCO Nod signals. The surprising thing about DB46 is that it not only possesses lectin activity but it is also a nucleotide phosphohydrolase (i.e., apyrase). Moreover, Etzler et al. (1999) showed that addition of LCO to purified DB46 significantly enhanced apyrase activity. Therefore, DB46 represents a promising candidate for a Nod signal receptor that could, in the bound state, activate cellular processes via its apyrase activity.

Identification of the Nod signal receptor is clearly important. However, equally important is understanding how Nod signal perception induces nodule formation. Elucidating these steps will involve the use of cell biology methods. What is needed is new methods that will allow investigators to investigate these downstream events. The Spaink laboratory is investing a great deal of effort in developing sophisticated fluorescent methods to explore Nod signal binding and signaling events.

One of the clear results of Nod signal action on the plant host is the initiation of cortical cell division. This involves modulating the plant cell cycle and the induction of meristematic activity. The Kondorosi laboratory is focusing on exploring these methods as a route to understanding Nod factor signaling. Pharmacology methods, being used by several laboratories, are also being used to decipher what cellular signal pathways are involved. This work has implicated such things as inositol phosphates, G proteins, protein kinases, etc. in Nod factor signaling. These data suggest similarities between nodule initiation and other, well characterized cell signaling pathways. The challenge will now be to identify the specific similarities and differences found in the Nod signal pathway.

The cell biological methods being developed in the Kondorosi and Spaink groups are examples of similar efforts taking place in a number of laboratories. Ultimately, these efforts will provide the tools to dissect the Nod signal pathway into its individual steps. As in the past, work on the rhizobial-legume symbiosis will benefit from the application of a variety of genetic, molecular, biochemical and cell biological approaches. In this regard, legume nodulation continues to provide a model for sophisticated investigations of plant development.

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PERCEPTION OF LIPOCHITOOLIGOSACCHARIDIC NOD FACTORS IN THE LEGUME-*RHIZOBIA* SYMBIOSIS: STUDIES ON *MEDICAGO* NOD FACTOR BINDING SITES

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1. Introduction

The *legame-rhizobia* symbiosis is characterised by the specificity of interaction between the prokaryotic and eukaryotic partners. Although some rhizobia species are able to nodulate a large number of hosts, many of them are restricted to interacting with only a few genera of legumes (for reviews see Long, 1996; Denarié et al. 1996; Schultze, Kondorosi 1998; Albrecht et al. 1999). This specificity of host-partner recognition, associated with the morphogenesis of a new plant organ are fundamental characteristics of this nitrogen-fixing symbiosis. It is now known that these characteristics are initiated by an exchange of signals between the compatible partners. The plant root exudates contain elicitors, normally flavonoids, which induce the expression of the *nod* genes, the rhizobial determinants of nodulation and host-range. These genes encode mainly enzymes involved in the synthesis of the return signal - the Nod factors. These factors are lipochitooligosaccharides (LCOs) which are recognised by the legume partner and elicit several of the early steps of rhizobial nodulation, and in some legumes even the triggering of the whole nodule morphogenic programme. The specificity of the interaction is ensured by the exact chemical structure of the signals and the ability to recognise them. Although much is known of the structure and perception of the plant signal and the structure of the bacterial signal, only rudimentary knowledge has so far been obtained on the mechanism of perception of the Nod factors by the plant hosts. How these LCOs are perceived is thus the next major challenge in our understanding of host specificity and nodule organogenesis in the symbiosis.

To date most information on Nod factor perception has come from studies of plant symbiotic responses, using both plant and bacterial mutants and differently substituted Nod factors (Long, 1996; Denarié et al. 1996; Conn et al. 1998; Albrecht et al. 1999). The symbiotic association between *Sinorhizobium meliloti* and *Medicago* plants has been the best studied and this is likely to continue with the complete sequencing of the tripartite *S. meliloti* genome and the adoption by the world-wide symbiotic community of *M. truncatula* as a model legume for genomic and genetic studies. *S. meliloti* produces a family of closely related sulphated Nod factors of which the major one is NodRm-IV (Ac, S, C16:2). Studies using *S. meliloti* mutants defective in *nod* genes, that alter one or more of the substitutions has led to the suggestion that Nod factors are perceived by two different receptors, one responsible for entry of the bacteria in which the structure of the fatty acid and the presence of the O-acetate are important and a second which is less stringent and involved in signalling (Ardourel et al. 1994). Both receptors are envisaged to perceive sulphated factors, a suggestion supported by the dependence of all *Medicago* symbiotic bioassays on sulphated factors (Truchet et al. 1991; Journet et al. 1994). The idea of dual Nod factor receptors is supported in soybean by studies on purified LCOs (Minami et al. 1996) and in pea, by studies of the plant *SYM2* mutant (Geurts et al. 1997).

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A variety of approaches are now being used to study more directly the perception of Nod factors by the plant. Firstly Nod factors and their binding sites are being localised in plants using cytology. Both fluorescent factors and antibodies to Nod factors are available and have been used for this work (Philip-Hollingsworth et al. 1997; Timmers et al. 1998; Gadella et al. 1999). Secondly a genetic approach is being used to identify loci which may be involved in Nod factor perception. Generally two phenotypes are being searched for: Nod$^-$ indicating loss of a key component for nodulation (such as a Nod factor receptor) and alteration of partner specificity dependent on Nod factor structure. The *SYM2* mutant falls into the latter category (Albrecht et al. 1999) and recently other genes have been described in *M truncatula* (T. Huguet, J. Dénarié, personal communication). Thirdly a biochemical approach is being used to identify Nod factor binding proteins in legumes which may be candidates for receptors. In this article we will describe our studies using such an approach in *Medicago.*

2. Nod Factor Binding Sites in *Medicago*

In order to search for receptor sites which are often of low abundance and high affinity, a ligand is required which both binds to the sites and which can be detected with high sensitivity. For this purpose we have used only ligands corresponding to the major Nod factor of *S. meliloti,* which have been shown to be biologically active and hence must be recognised by the plant roots. Secondly these molecules have been labelled to a high specific radioactivity either chemically with tritium in the fatty acid or enzymatically with ^{35}S-sulphate on the reducing sugar, resulting in ligands exhibiting specific radioactivities of 56 and 800 Ci/mmol respectively (Bono et al. 1995; Bourdineaud et al. 1995). The availability of two ligands each labelled in a different moiety of the Nod factor has also allowed us to verify that a binding site binds the whole LCO molecule.

Initially, as the source of biological material, we used roots from nitrogen-starved *M truncatula* plants which were thus competent to interact with rhizobia and Nod factors. Extraction and differential centrifugation led to a number of fractions that were tested for binding to the tritiated ligand (Bono et al. 1995). The low speed (3000g) fraction showed the highest specific binding and a site was characterised which has been termed Nod Factor Binding Site 1 (NFBS1). This low speed fraction is likely to contain cell wall and nuclear debris and some associated or trapped membranes. The binding to this fraction was characterised in terms of affinity and specificity and showed that the site is clearly a site for LCOs as chitin fragments and palmitate are

Table 1. Comparison of Nod Factor Binding Sites (NFBSs) in *Medicago*.

Property	NFBS1	NFBS2
Plant material	*M. truncatula* roots	*M. varia* cell cultures
Extract fraction	3000g	45000g
Subcellular location	Unknown	Plasmamembrane +?
Protease sensitivity?	Yes	Yes
Characterising ligand	NodRm-IV (Ac, S, C16:2-^{3}H)	NodRm-IV (Ac, ^{35}S, C16:2)
Affinity (K_d) for ligand	86 nM	4 nM
Affinity (K_d) for tetra-N-acetyl-chitotetraose	100 µM	2 µM
Selectivity	LCOs	LCOs with certain substitutions

poor competitors of ligand binding and exhibits an affinity (K_d) of about 80 nM for the Nod factor (Table 1).

Secondly we used M *varia* cell suspension cultures. Although not a symbiotic material, we have used cell cultures as they often express receptors at a higher abundance than in natural conditions and are well suited to biochemical studies. In this material most of the specific Nod factor binding occurred in the 45000g or 'microsome' fraction. Further characterisation suggested that this fraction contains two different binding sites; a site similar in affinity to NFBS1 and a second higher affinity binding site which has been termed NFBS2 (Niebel et al. 1997). Both sites are protease sensitive. The occurrence of similar affinity sites in the 3000g fraction of *M truncatula* roots and the 45000g fraction of *M varia* cell cultures may suggest that i) an essentially identical site is either located in different subcellular compartment in the two materials or ii) that the same compartment behaves differently in the two extracts or iii) that the sites are structurally and geographically distinct but happen to have similar thermodynamic characteristics. At present we are unable to distinguish between these possibilities.

The thermodynamic characteristics and selectivity of NFBS2 have been determined recently (Gressent et al. 1999). This site has a higher affinity for the ligand (K_d of about 4 nM) and exerts certain structural requirements for Nod factor recognition. Like NFBS1 it is clearly a site for LCOs rather than COs. However binding to NFBS2 is highly dependent on the nature of the substitutions on the non-reducing sugar: the structure of the fatty acid on C_2, the presence of the O-acetate group on C_6, and a hydroxyl group in an equatorial rather than axial position on C_4. The sulphate group whether on the 4^{th} or 5^{th} terminal reducing sugar is not discriminated. However the reducing sugar seems to interact with NFBS2 because pentameric, rather than tetrameric, rather than trimeric factors, bind with higher affinity and reduction of the anomeric carbon drastically reduces the binding.

3. Putative roles of *Medicago* NFBSs

NFBS1 is clearly the most abundant Nod factor binding site in roots of *Medicago* but a site with similar characteristics also exists in tomato roots (Bono et al. 1995). The presence in a non-legume suggests that these sites have a wider role than in the symbiosis. Whether it acts normally as a binding site for LCOs, perhaps of plant origin, or has some other role and happens fortuitously to bind Nod factors, can only be speculated upon. In *Medicago* its subcellular and tissue localisation needs to be determined in order to assess whether it is expressed in a position likely to encounter Nod factors. In view of the recent localisation of fluorescent Nod factors in the cell walls of *Vicia sativa* (T. Gadella, personal communication) it is tempting to speculate that NFBS1, which has been characterised in the 3000g fraction of the extract, may account for cell-wall binding. Further experiments are clearly required to determine this.

NFBS2 is a much higher affinity site and, at least partially, located in the plasmamembrane of cell cultures. A site of similar affinity occurs in the microsomal fraction of M *truncatula* roots but its abundance is too low to determine whether it has the same selectivity as NFBS2 (F. Gressent and J.-J. Bono, unpublished). A high affinity plasmamembrane LCO binding protein which partially discriminates the structural decorations on Nod factors could reasonably be expected to play a symbiotic role if it were expressed at a suitable time and location. Whether it would then act in transport, sequestration or signal transduction is another question. Certain characteristics of this binding site are consistent with a role in signal perception: high affinity and reversible binding, high degree of selectivity. However NFBS2 is unable to discriminate the sulphate group which is the major determinant of nodulation, host range and all symbiotically-relevant bioassays in *Medicago*. It is noteworthy though that non-sulphated Nod factors are active in a root hair alkalinization bioassay and appear to be recognised in a manner different to sulphated factors (Felle et al. 1996).

If NFBS2 were involved in symbiotic Nod factor perception, it could act according to different scenarios (Fig. 1). Firstly it may interact with an adaptor protein that discriminates the sulphate group (A). Secondly it may act in parallel with another receptor that is able to discriminate the sulphate (B). Thirdly the specificity for the sulphate may lie upstream of this receptor, perhaps in the selective degradation of non-sulphated factors (C). This hypothesis is suggested by the work of Staehelin et al. (1994) who have shown that sulphated factors are more stable on *Medicago* roots than non-sulphated ones.

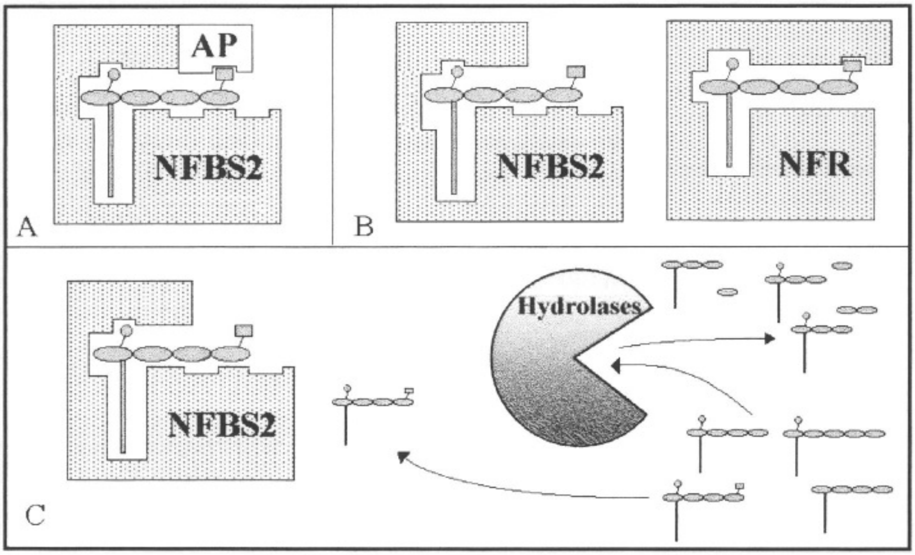

Fig. 1. Hypothetical models of Nod factor perception by *Medicago* Nod Factor Binding Site 2 (NFBS2) and selectivity for sulphated factors. A. The selectivity may be determined by an adaptor protein (AP) directly associating with NFBS2. B. NFBS2 may be a minor receptor working in parallel with a major Nod factor receptor (NFR). C. Hydrolases degrade non-sulphated factors so that only sulphated ones are available for perception, by a receptor that does not need to be selective.

4. Towards the structural identity of NFBSs

As illustrated above it is difficult to assign physiological and cellular roles to proteins that have only been characterised biochemically in extracts. Even more so when the extracts are of cell cultures which are not relevant to the physiological situation. The characteristics of NFBS2 suggest however that it may at least be structurally related to symbiotic receptors. For this reason we are pursuing the cloning of its genes in order to use the tools of molecular and reverse genetics to assess the role of NFBS2 and related proteins in the symbiosis. To date we have succeeded in solubilising NFBS2 with a 75% recovery of binding activity for 18% of the microsomal proteins. This fact augers well for the possibility of purifying the protein and obtaining a partial protein sequence.

Recently the group of M. Etzler have reported the characterisation and cloning of a novel lectin from the legume *Dolichos biflorus* which shows a specificity for Nod factors (Etzler et al. 1999). This protein also has an ATPase activity of the apyrase type and was therefore called lectin nucleotide phosphohydrolase (LNP). Anti-LNP antibodies have localised LNP to the surface of root hairs and have been shown to interfere with responses to the rhizobial symbiont, thus suggesting that this protein plays a role in symbiosis. Recently we have isolated clones from *M. truncatula* related to this apyrase and are presently testing whether the encoded proteins bind *S. meliloti* Nod factors and may account for either of our binding sites.

5. Conclusion

Revealing the mechanisms of perception of Nod factors is the next great challenge in a quest to understand the determination of partner specificity and subsequent nodule organogenesis in the legume-rhizobia symbiosis. Our biochemical approach to this problem has revealed that *Medicago* contains at least two high affinity proteins for LCOs (related to NFBS1 and NFBS2) but it remains to be seen whether they correspond to putative receptors for the symbiotic Nod factor signals.

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7. Acknowledgements

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USE OF GFP TO STUDY FACTORS INVOLVED IN THE *LOTUS JAPONICUS* SYMBIOSIS

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1. Introduction

Lotus japonicus has been shown to be a very suitable model system to study nodulation and nitrogen fixation (Handberg, Stougaard 1992). We are currently using this model system to study in detail the infection and nodulation process induced by *Mesorhizobium loti.* To this end, a number of marker lines of *L.japonicus* have been constructed. For instance, fusions of various promoters to the green fluorescent protein (GFP) gene have been obtained. These promoters are either known promoters that were selected based on their known responsiveness to Nod factors or plant hormones or they were obtained as a result of a random promoter trapping approach as outlined in this paper. In addition, new methods for analysis of calcium fluxes and changes in the cytoskeleton based on fluorescence microscopy are being tested. Using these technologies, we analyze the function of host-specific modifications of the Nod factors. Below, we briefly describe our results on the analysis of the role of the acetylfucose residue of the Nod factors of *M. loti.*

2. A *Lotus japonicus* nodulation system based on heterologous expression of the fucosyl transferase NodZ and the acetyl transferase NolL in *Rhizobium leguminosarum*

Heterologous expression of NodZ and NolL proteins in *R. leguminosarum* bv. *viciae* led to the production of acetyl fucosylated lipo-chitin oligosaccharides (LCOs), indicating that the NolL protein obtained from *Mesorhizobium loti* (Scott et al. 1996) functions as an acetyl transferase. These results confirm published observations on the function of NolL in other rhizobia (Berck et al. 1999; Corvera et al. 1999). Efficient nodulation of *Lotus japonicus* by the NodZ/NolL-producing strain was demonstrated. Nodulation efficiency was further improved by the addition of the ethylene inhibitor AVG. The obtained efficient heterologous nodulation system for *L. japonicus* can be used for the analysis of factors other than the LCOs involved in infection and nodulation. For instance, our test system can be used to analyze the effect on nodulation of *L. japonicus* of well-characterized mutations in *R. leguminosarum* genes, for instance those involved in EPS biosynthesis or avirulence (e.g. see Pollock et al. 1998; Roest et al. 1997).

3. The use of GFP as a viable marker for analysis of the infection and nodulation processes

The protein called green fluorescent protein (GFP), was isolated from the jellyfish *Aequorea victoria,* shows green fluorescence after blue light excitation (e.g. see Tsien 1998). The gene for this protein has been cloned and when expressed in heterologous systems leads to the production of an auotfluorescent protein. Therefore, autofluorescent derivatives of a protein of interest can be obtained by fusion of their genes with the gene encoding GFP. Various derivatives of GFP optimised for expression and shifted in excitation and emission spectra have been constructed which are generically called autofluorescent proteins (AFPs). The three most popular of them are the proteins CFP (430 nm excitation, 480 nm emission), eGFP / sGFP (480 nm excitation, 515 nm emission) and YFP (515 nm excitation, 545 nm emission). The GFP protein and its various

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derivatives have been used extensively to study many biological processes such as transcription, translation, translocation and interactions of proteins in living cells.

For the analysis of promoter activity, it is often very useful to make use of a fusion construct consisting of the GFP and 8-glucuronidase (GUS) (Quaedvlieg et al. 1998). Using this construct we have obtained various transgenic *L. japonicus* lines which will be used for analysis of responses to LCOs. For instance, we have fused the GH3 promoter to the GFP-GUS reporter construct in order to analyse the effect of LCOs on auxin transport in *L.japonicus* using microtargeting and following the strategy of Mathesius et al. (1998). A novel application of the GFP protein has come from the studies from the group of Tsien (Miyawaki et al. 1997). These authors have constructed a calcium sensor protein called "Cameleon" which is based on the phenomenon of fluorescence resonance energy transfer. This construct was recently shown to be applicable in plants (Gadella et al. 1999). For the analysis of GFP various microscopic imaging techniques have been employed in our laboratory. The most commonly used technique is confocal laser scanning microscopy (CLSM) which enables the four dimensional (i.e. in space and time) visualization of GFP in living tissues. However, a pitfall that is often encountered is the high level of autofluorescence in various tissues (e.g. in plants). This problem can in several cases be solved with the use of spectral resolved CLSM as is possible with the new Leica TCS SP system. However, in difficult cases where there is low expression of the autofluorescent proteins, use has to be made of fluorescent lifetime imaging techniques. An important new application for the study of autofluorescent proteins comes from the use of multiple-photon CLSM. This technique is based on the principle that UV or visible fluorochromes can be excited by simultaneous excitation with multiple photons of near infrared light frequency. Two-photon microscopy has the following advantages: (1) Since there is no need for a pin-hole, higher light intensities are obtained. (2) Photo-damage as compared to UV and visible laser illumination is minimal (restricted to thermal damage) (3) Bleaching of the fluorophore is limited to the time that a single point is scanned (since the fluorophore is excited only at the focal point) (4) near infra red light travels deeper into tissues. This is especially important for the analysis of plant tissues which have thick cell walls. (5) Pulsed lasers can also be efficiently used for measurements of fluorescent lifetimes (e.g. see Gadella 1997).

4. Promoter trapping in *Lotus japonicus.*

In order to find plant genes responding to the application of rhizobia or (derivatives of) LCOs to roots, a promoterless reporter gene consisting of the *gusA:intron/gfp* fusion gene (Quaedvlieg et al, 1998) was inserted into the genome of *Lotus japonicus* (promoter trapping) using *Agrobacterium*-mediated hypocotyl transformation (Handberg et al. 1994). It might be envisaged that in this way novel LCO-responsive plant genes might be isolated. Such genes are either specific for the *Rhizobium-plant* interaction or they have a general function in cell development. An advantage of this strategy is that the promoters of interest are analysed in their natural context, i.e. including all its upstream and downstream regulatory elements. To enable isolation of plant DNA sequences flanking the T-DNA insert through plasmid rescue, a pUC plasmid sequence was inserted between the T-DNA borders as well (Figure 1).

The feasibility of this approach was tested in a pilot experiment in which 130 independent transgenic lines were generated. All of them had a normal phenotype and set seed except for one line that showed an altered leaf and root morphology and did not flower. Half the amount of the primary transformants was assayed for GUS expression in different parts of the plant (leaf, stem, root, nodule, flower) and approximately 20% of the plants showed GUS expression to some extent.

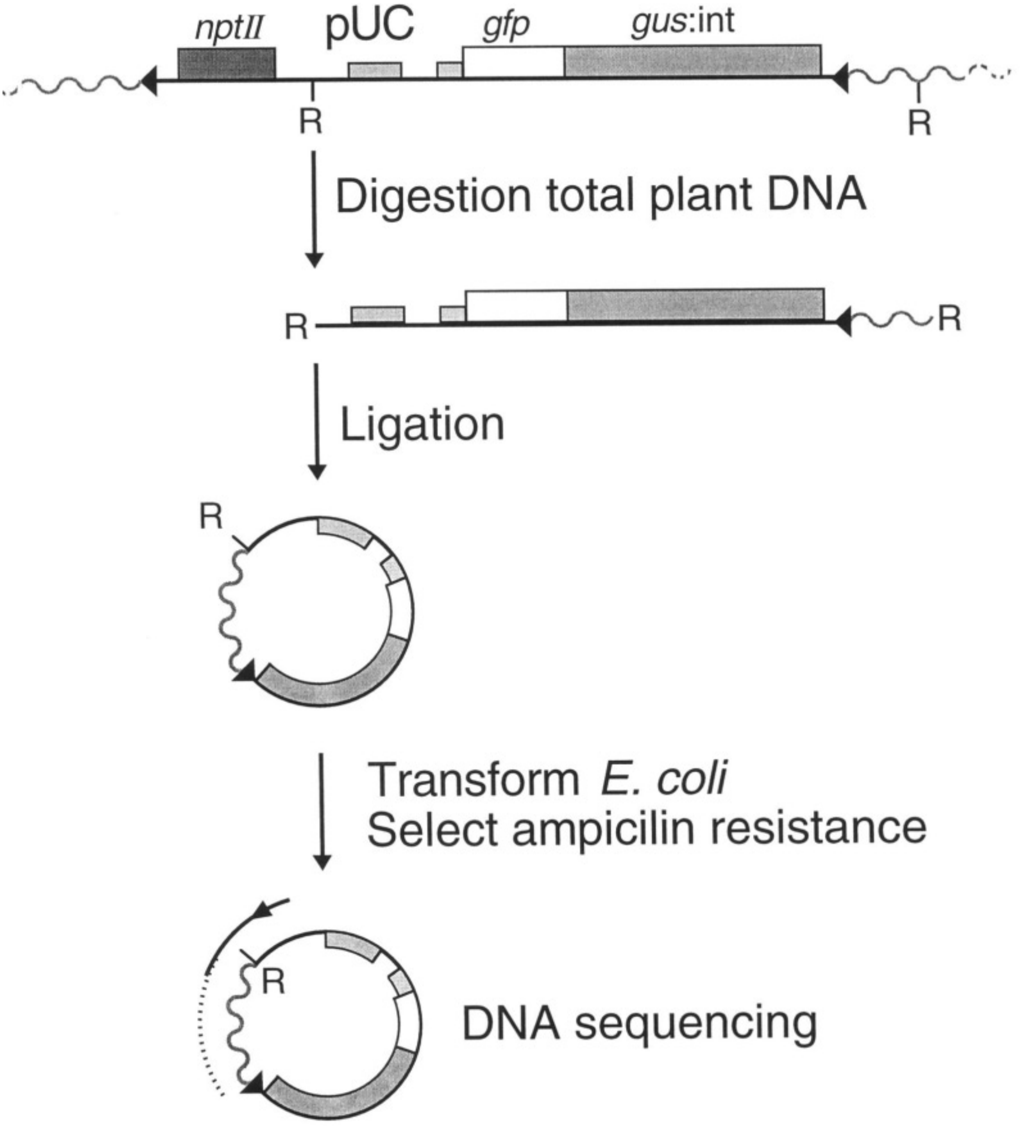
nptII
pUC
gfp
gus:int
R
R
Digestion total plant DNA
R
R
Ligation
R
Transform E. coli
Select ampicilin resistance
R
DNA sequencing

Figure 1: Schematic diagram showing the principle of plasmid rescue to isolate plant DNA sequences flanking the promoter trap T-DNA construct.

The T2 progeny was generated, inoculated with *Mesorhizobium loti* and 10-14 days later GUS expression was determined. Approximately 10% of the seedlings examined so far showed a nodule-specific expression of the reporter gene, a surprising high frequency. For example, line *Lj2B* expresses the reporter gene in the vascular bundle of the root and stem but the expression is enhanced at the position where the nodule emerges from the root. Line *Lj*2184.145 is another example of nodule-specific expression where the reporter gene is expressed at very low levels in a relative young part of the root and expressed at very high levels in a characteristic pattern in the nodule. The expression in the nodule seems to decline with age.

Currently, we are finishing the analysis of the reporter gene expression in inoculated T2 seedlings of our complete collection of promoter trapped lines. Moreover, it will be tested whether nodule or nitrogen fixing mutants will segregate. The most interesting lines will be analysed in detail to obtain information about for instance tissue specificity of expression, and plant DNA sequences that drive the expression of the reporter gene will be isolated and analysed. Our data clearly show that promoter trapping in the model legume *L. japonicus* is a fruitful approach to identify genes involved in nodulation and analyse their expression in detail.

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6. Acknowledgements

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CELL CYCLE CONTROL IN ROOT NODULE ORGANOGENESIS

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1. Introduction

The Nod factors are mitogenic signals that trigger cortical cell division in legume roots grown under nitrogen-limiting conditions. Nodule organogenesis is restricted to the emerging root hair zone where cortical cells located opposite to the protoxylem poles are transiently competent for Nod factor-activated cell division. This positional information is determined by gradients of various endogenous plant hormones (auxin, cytokinin, ethylene) and other growth stimulating factors (Schultze, Kondorosi, 1998). In plant cells, auxin and cytokinin play essential roles in cell cycle regulation by controlling the activity of cyclin-dependent kinase (Cdk) - cyclin complexes. Auxin induces the expression of *cdk* genes and it is required during the cell cycle, whereas cytokinin controls G2-M transition by activating the mitotic Cdks (Zhang et al. 1996) but it is also required for the expression of certain D-type cyclins (Sony et al. 1995). Nod factors act on the GO-arrested cells, however, only the inner cortical cells undergo cell division, while the outer cortical cells become arrested in G2 (Savouré et al., 1994; Yang et al., 1994). In *Medicago,* cytokinin seems to play a pivotal role in nodule initiation. When plants are grown in nitrogen-limiting conditions exogenously supplied cytokinin (benzylaminopurine) can also induce cell divisions and in some cases even formation of pseudonodules (Cooper, Long, 1994; Bauer et al. 1996; Fang, Hirsch, 1998). The Nod factor-inducible early noduline genes tested (*Msenod12a, Msgbl, Mscal, Msenod40*) were induced also by cytokinins (Bauer et al. 1996; Coba De la Pena et al. 1997; Crespi et al. 1997; Fang, Hirsch, 1998). Thus, cytokinins and Nod factors may coordinate the control of cell division in the root cortex and the elicitation of common biological responses suggest the convergence of Nod factor- and cytokinin-signalling pathways or at least some of their elements.

2. Reactivation of the Cell Cycle in the Root Cortex

Cell cycle studies in *Medicago* species, the symbiotic partners of *Rhizobium meliloti,* resulted in the identification of a set of cell cycle marker genes specific for different phases of the cell cycle including CDKs, Gl-type and mitotic cyclins and S-phase-specific histones such as H3 which made possible to investigate the order of cell cycle events evoked by Nod factors or rhizobia (Fig. 1).
Our data indicate that activation of the *cycMs3* and *cycMs4* cyclins (Meskiene et al. 1995; Dahl et al. 1995) in the nodulation competent root zone might be the earliest cell cycle responses elicited by Nod factor. Stimulated expression of *cycMs3* classified as *CycA2* was observed within 1 hour while that of *cycMs4* could be detected only a few hours later. These results suggest that the level of *cycMs3* transcript might be involved in reactivating the cell cycle in the root cortex while the activity of the CycMs3-CDK complex might be required for that of CycMs4. CycMs4 is a D-type cyclin belonging to the D3 group of plant cyclins (Dahl et al. 1995). From M. *sativa* we have isolated two cyclin D3 genomic clones exhibiting different patterns of expression by RT-PCR analysis. *CycD3-I* expressed transiently, usually from 9 hours until the first cell divisions. In contrast, the expression of *CycD3-II,* was induced when the nodule primordium started to differentiate to distinct zones. Thus, *CycD3-I* might be involved in the recruitment of cortical cells while *CycD3-II* may play a role in the differentiation of symbiotic cells.

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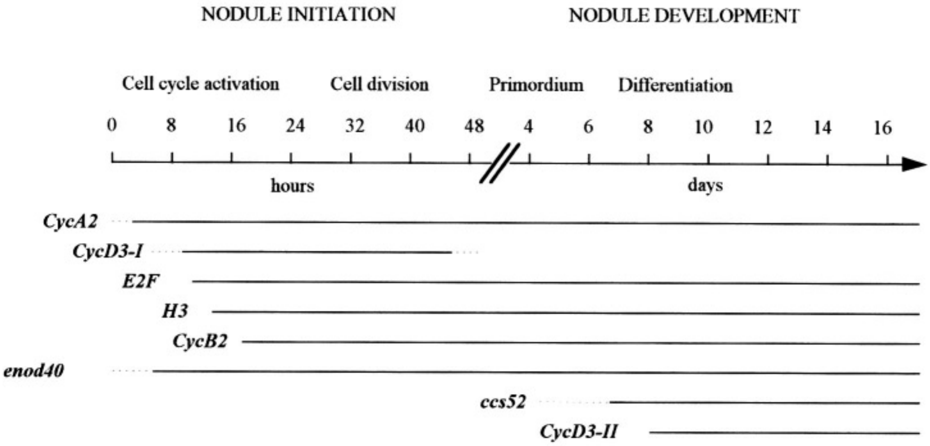

Figure 1. Expression of cell cycle genes and *enod40* during nodule development

In mammals, the key function of the cyclin D-dependent kinases is the phosphorylation of the retinoblastoma (Rb) protein and liberation of the E2F transcription factors from the inactive Rb-E2F complex for transcription of genes needed to initiate S phase. From *M. truncatula* we identified 2 members of the E2F family, both of which were induced in the roots 9-12h after the application of Nod factors or rhizobia, consistently with the expected timing of Gl/S transition. G2-M transition is controlled by the activity of the mitotic cyclin-CDK complexes referred to as MPF (M-phase/maturation promoting factor). By Nod factor-treatment, the *Medicago* B-type mitotic cyclins were induced later than the Gl- and S-phase specific marker genes (Savouré et al. 1994). At present, it is unknown why mitotic cyclin expression is restricted to the inner cortex. One can speculate that cyclin B expression might be under phytohormonal control and hormone gradients might be different in the outer and inner cortical cell layers.

3. Under Nitrogen-limiting Conditions, Expression of the *enod40* Gene Induces Cortical Cell Division

The *enod40* gene is expressed early at the onset of *Medicago* root nodule organogenesis (Fig. 1) and it is induced by Nod factors and cytokinins as well. It codes for an unusually small peptide (12 or 13 amino acids) (van de Sande et al., 1996) and contains a conserved long 3' untranslated region that is able to form stable secondary structures (Crespi et al. 1994). Interestingly, the amounts of oligopeptides detectable in cells containing high levels of the *enod40* transcript are extremely low (van de Sande et al. 1996). To study the possible role of this peculiar gene during nodule development, we set out to construct transgenic *Medicago* plants overexpressing *enod40* from the 35S promoter. Using the efficient regeneration and transformation protocols developed for the diploid autogamous *M. truncatula* (Hoffmann et al., 1997; Trinh et al., 1998), we obtained transgenic lines overexpressing the *Mtenod40* gene at a relatively high level (Charon et al. 1997). Under nitrogen-limiting conditions, overexpression of *enod40* in these plants provoked more cortical cell division than in the control transgenic plant roots. Analysing a series of transgenic plants and their descendants, the *enod40* transcript levels and the number of cell divisions directly correlated. Using alfalfa plants carrying a fusion of the *Msenod12A* promoter to the *uidA* reporter gene (Bauer et al. 1996), delivery of the *enod40* expression cassettes to the roots grown under nitrogen-limiting conditions has led to a significant increase in the division of the inner cortical cells (Charon et al. 1997).

Transgenic *M. truncatula* plants overexpressing *enod40* exhibited accelerated nodulation by *R. meliloti.* This was due to increased initiation of primordia which was accompanied by proliferation response of the region close to the root tip and enhanced root length. The root cortex of the *enod40* transgenics showed increased sensitivity to Nod factors. Tl and T2 descendants of two transgenic lines with reduced level of *enod40* transcripts (likely due to co-suppression) formed only few and modified nodule-like structures (Charon et al. 1999).

Our results suggest that induction of *enod40* is a limiting step of the primordium formation and its function is required for appropriate nodule development. These results indicate again that *enod40* is one of the elements involved in the control of nodule initiation. It is likely that it is a component of the Nod signalling pathway leading to cortical cell division. The observations that the increase of cortical cell division was detectable only in roots under the physiological conditions permitting nodulation and only in the inner cortical cells, do indicate that the cortical cell division induced by *enod40* is controlled by a number of other plant factors acting on these cells independently from *enod40.* The *enod40* is the first nodulin gene which has been shown so far to act as a regulator of nodule organogenesis.

4. Differentiation of Nodule Cells: CCS52 Mediates Cell Division Arrest and Endocycles

As cell division foci in the root cortex develop to persistent meristems, cells start to exit from the mitotic cycle and enter differentiation continuously, leading to the development of indeterminate nodules. This nodule type is under a dynamic development and, except for the *de novo* meristem formation, the different stages of development can be monitored within a single nodule. Most of the cells undergo endocycles and increase the nuclear and cell volumes. In the internal nodule tissues, these events occur gradually in zone II where cell size increments could be particularly important to provide space for the multiplying bacteria and to host the enlarged bacteroids in the nitrogen-fixing stage of symbiosis. The direct correlation between endoreduplication and cell size suggests that endoreduplication might be necessary to increase the cell volume. Moreover, amplification of the genome by endocycles (from 2C up to 64C) might ensure the required dosage of genes involved in the symbiotic processes and in the altered metabolism.

Factors controlling transition from mitotic cycles to differentiation programs or conversion of mitotic cycles to endocycles have not been identified yet in plants. By screening a young nodule cDNA library for genes involved in nodule organogenesis in *M. sativa,* we identified a cDNA, *ccs52* which exhibited highly stimulated expression in the nodules compared to roots (Cebolla et al. 1999). Sequence analysis revealed that *ccs52* codes for a 52 kDa WD-repeat protein that is involved in ubiquitin-dependent proteolysis of mitotic cylins. Yeast and *Drosophila* homologs act as substrate-specific activators of the APC-dependent protein degradation pathway and are associated with differentiation processes; exit from the cell cycle and generation of endocycles (Sigrist, Lehner, 1997; Zachariae et al, 1998; Yamagushi et al 1997). Overexpression of *ccs52* in fission yeast resulted in the degradation of the fission yeast mitotic cyclin, CDC 13 leading to growth arrest, endocycles as well as elongation and enlargement of cells (Cebolla et al. 1999).

In *Medicago* nodules, expression of *ccs52* was localized in zone I where cells exit from proliferation and in zone II which is the major site for endoreduplication and cell differentiation. Northern analysis demonstrated that the *ccs52* transcripts were present also in other tissues indicating that the *ccs52* function is not restricted to nodulation and could be involved in the development of other polyploid organs. This was tested in transgenic *Medicago* plants overexpressing the *ccs52* cDNA in antisense orientation from the 35S promoter where downregulated expression of *ccs52* resulted in reduced degree of ploidy in the polyploid organs such as the petioles, roots and hypocotyls. Moreover, reduced ploidy correlated with the formation of smaller cells demonstrating that the expression level of *ccs52* affects directly both the degree of endoploidy and the size of the cell volume (Cebolla et al. 1999). Endocycles are particularly widespread in plants and the presence of *ccs52* homolog sequences in a wide variety of plant species indicates that *ccs52* might be a conserved regulator of the plant cell cycle having a fundamental role in differentiation programs.

5. Conclusions

Studies on root nodules shed light on novel functions of known cell cycle genes (eg. *CycD3, CycA2*) and resulted in the identification of new plant cell cycle regulators (eg. E2F, *ccs52, enod40*). For example, CCS52 that may play a general role in plant development by stopping cell division and transforming the mitotic cycle to endocycles was discovered from nodules probably because endoreduplication cycles persist and reach a high degree of polyploidy in nodules. Although the function of *enod40* remains a puzzle, existence of *enod40* homologs in non-legumes plants indicates that it might have a broader implication in plants and the knowledge gained on nodules could be extended for other plants.

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NOD FACTOR RECEPTION

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1. Introduction

Rhizobial Nod signals are substituted chitin oligomers, usually of four to five *N*-acetylglucosamine (GlcNAc) residues, mono-*N*-acylated at the non-reducing end and carrying a variety of substitutions at both the reducing and non-reducing terminal GlcNAc residues. Each rhizobial species produces a variety of Nod signals with specific substitutions (Denarie et al. 1996). Nod signal structure does not correlate with rhizobial phylogeny but with the host range of the particular rhizobium. This suggests that each host plant has evolved to recognize the specific Nod signal produced by its compatible symbiont. Inoculation of roots with the appropriate Nod signal will elicit many of the responses seen upon rhizobial inoculation (reviewed in Cohn et al., 1998). The fact that Nod signals act at very low concentrations and exhibit chemical specificity suggests that perception is likely mediated by specific protein receptors. Table 1 summarizes what is known about lipo-chitin and chitin binding proteins in plants. In the case of legumes, these binding proteins represent candidates for a Nod signal receptor. To better define the specificity of Nod signal action on soybean, we undertook structure/function studies using a variety of natural and synthetic LCO. For example, Stokkermans et al. (1995) demonstrated that only four LCO [i.e., LCO-V(C18:1Δ11, MeFuc), LCO-V(C18:1Δ9, MeFuc), LCO-V(C16:0, MeFuc), and LCO-IV(C16:0)] could elicit a morphogenic response on soybean roots. These studies demonstrated that fucosylation and chitin chain length were the most important determinants for Nod signal specificity.

We extended this work by using the expression of early nodulin genes as a measure of Nod signal activity. Early nodulins are plant proteins whose expression is specifically enhanced early (within 48 hours) after rhizobial inoculation. Minami et al. (1996a) showed that expression of the early nodulin ENOD40 could be induced transiently by a simple, non-acylated chitin pentamer. However, sustained expression of ENOD40 required a soybean-specific Nod signal (i.e., one of the four LCO listed above). In a subsequent paper, Minami et al. (1996b) showed that the addition of any single LCO to soybean roots failed to elicit the expression of the early nodulin ENOD2. However, when a mixture of LCO was added, ENOD2 expression was induced. Analysis of these mixtures demonstrated that addition of a non-acylated, chitin pentamer, along with one of the four, morphogenic LCO molecules, was sufficient to induce ENOD2 expression. These studies by Minami et al. (1996a,b) pointed to the presence of two Nod signal recognition events in soybean that vary in their chemical specificity and are independently coupled to cellular events. Since expression of ENOD40 and ENOD2 mark different stages in nodule ontogeny, these results suggest that different Nod signal recognition events are required nodule development. A low-stringency recognition step is requiredc to initiate some of the first steps in nodule ontogeny (e.g., transient ENOD40 expression) but is not sufficient to sustain nodule development. Likewise, activation of a high-affinity recognition step results in sustained ENOD40 expression and the formation of an incomplete, nodule primordium. However, only when both of these recognition events are

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simultaneously activated, does nodule development proceed to the formation of vascular tissue and the expression of ENOD2.

Table 1. Summary of plant lipo-chitin and chitin binding proteins.

Trait	Legumes	Rice	Soybean	Tomato
Chitin	*Lipo-chitin oligomer*	*Chitin oligomer*	*Chitin oligomer*	*Chitin oligomer*
Oligomer size	dp= 4 -5	dp= 7-8	dp= 7-8	dp= 4
Chitin-binding protein, molecular weight	*Alfalfa*, unknown Apyrases: *D. biflorus*, 46 kDa (Etzler et al., 1999); *G. soja*, 50 and 52 kDa[1] (Day et al., unpubl.); *M. truncatula*, approx. 46 kDa[1] (Cohn et al., unpubl.).	75 kDa (reviewed in Stacey, Shibuya, 1997)	~85 kDa (Day et al., unpubl.)	unknown
Dissociation constant	*Alfalfa:*NFBS1,Kd = 72 nM NFBS2, Kd= 2 nM (Bono et al., 1996) Apyrases: unknown	Kd= 29 nM (Shibuya et al., 1996)	Kd= ~50 nM (Day et al., unpubl.)	Kd= 23 nM (Baureithal et al., 1994)
Cellular Location	*Alfalfa:* Root, membrane fraction (Bono et al., 1996) Apyrases: *D. biflorus*, root, soluble and on root hair surface (Etzler et al., 1999) *G. soja* and *M. truncatula*, unknown	Plasma membrane (suspension culture cells) (Shibuya et al., 1996)	Plasma membrane (root and suspension culture cells) (Day et al., unpubl.)	Microsomal membranes (suspension culture cells) (Baureithal et al., 1994)
Cellular responses elicited by optimal chitin oligomer	*Lipo-chitin oligomer induces* (reviewed in Denarie et al, 1996; Cohn et al., 1998; Stacey, Shibuya, 1997): examples: -membrane depolarization -calcium spiking -gene expression	*Chitin oligomer induces* (reviewed in Stacey, Shibuya, 1997): examples: -membrane depolarization -gene expression -protein phosphorylation -phytoalexin production	*Chitin olgomer induces*: -ENOD40 expression (Minami et al., 1996a) -medium alkalinization (Day et al., unpubl.) -oxidative burst (Day et al., unpubl.)	*Chitin oligomer induces* (reviewed in Stacey, Shibuya, 1997): -medium alkalinization -protein phosphorylation

[1] Lipo-chitin binding activity of apyrases from *G. soja* and *M. truncatula* has not been shown experimentally and is based solely on the sequence similarity between these proteins and DB46 from *D. biflorus* (Etzler et al., 1999).

2. Chitin-binding Proteins

The ability of non-acylated chitin oligomers to induce ENOD40 expression in soybean suggested that a chitin binding protein could be involved in Nod signal recognition. Our hypothesis was that

Nod signal receptors could be a special class of chitin binding proteins found in a wide variety of plants. Therefore, we initiated a study to identify and characterize chitin-binding proteins in plasma membrane preparations from both soybean suspension cultured cells and root preparations (Day et al., unpubl.). These studies used ^{125}I-labeled conjugates of *N*-acetylchitooctaose and *N*-acetylchitopentaose as ligands. We were able to demonstrate saturable binding of both ligands to soybean plasma membrane preparations. Binding of chitin oligomers showed an apparent Kd of approximately 50 nM. The binding site has slightly better affinity for the *N*-acetylchitooctaose than for *N*-acetylchitopentaose. Indeed, competition experiments, using chitin oligomers (d.p.=2-8), demonstrated a preference for the higher molecular weight oligomers. In this regard, the binding site is almost identical to that reported for rice (Stacey, Shibuya, 1997). These data also suggest that the chitooctaose and chitopentaose are interacting with the same binding protein. Affinity labeling using a ^{125}I-labeled *N*-acetylchitooctoase ligand identified an 85 kDa protein. Addition of unlabeled ligand eliminated this binding indicating that the 85 kDa protein is a specific chitin-binding protein. The binding specificity of this 85 kDa protein for various chitin oligomers correlated well with the ability of the same oligomers to induce an oxidative burst response in soybean suspension cultured cells. Work to demonstrate the interaction of this binding protein with the *B. japonicum* Nod signals is continuing. At this point, it seems likely that this chitin-binding protein may be involved in the elicitation of a defense response to fungal pathogen cell wall fragments.

3. NOD Signal-binding Proteins

Lectins have long been implicated in the legume nodulation process but a precise role for these interesting proteins has not been defined. Recently, Etzler et al. (1999) reported the identification of a unique lectin (DB46) isolated from roots of the legume *Dolichos biflorus*. This lectin is not related to the common legume seed lectins. The *D. biflorus* lectin was found to bind to Nod signals from a variety of rhizobia. However, DB46 showed the highest apparent affinity for the Nod signal produced by *B. japonicum* and *Rhizobium* sp. NGR234, both of which can nodulate *D. biflorus*. Sequence comparisons revealed that the *D. biflorus* lectin was likely an apyrase (i.e., NTPase). Indeed, Etzler et al. (1999) demonstrated that the lectin possessed ATPase activity, which was significantly increased upon addition of the Nod signal. For this reason, the lectin was termed a lectin-nucleotide phosphohydrolase (LNP). The *D. biflorus* LNP (i.e., DB46) was found on the surface of root hairs using fluorescent antibody labeling. The properties of this protein suggest that it could play a role in Nod signal recognition and transduce this binding signal by way of its apyrase enzyme activity.

We have been exploring the possible role of apyrases in legume nodulation. For example, we have demonstrated the presence of orthologs of the *D. biflorus* LNP in both soybean and *Medicago truncatula*. Two soybean apyrase cDNA clones were isolated by PCR amplification using primers to conserved motifs found in all apyrase genes. The two soybean cDNAs are predicted to encode proteins of approximately 50 kDa (i.e., GS50) and 52 kDa (i.e., GS52). The genes encoding these proteins are differentially expressed in soybean. For example, both genes are expressed in roots and flowers, but only GS50 is expressed in stems. In roots, GS50 is constitutively expressed at a low level, while GS52 mRNA is rapidly induced upon *B. japonicum* inoculation. Therefore, GS52 classifies as an early nodulin. We have generated antibody to both GS50 and GS52 in order to localize the tissue and cellular expression of these proteins. Initial in vitro experiments demonstrate that both GS50 and GS52 possess apyrase (i.e., NTPase) activity.

We initially isolated only one full-length and one partial *M. truncatula* cDNA clone by PCR amplification. The full-length cDNA is predicted to encode a protein (i.e., MT46a) of approximately

46 kDa. Using a gene specific probe made from the 3' end of the cDNA, it was demonstrated that MT46 mRNA is induced very rapidly (i.e., within 3 hours) of inoculation with *Sinorhizobium meliloti.* Therefore, MT46a is also an early nodulin. The gene encoded by the partial cDNA clone has been termed MT46b.

We have screened a large collection of *M. truncatula* mutants defective in nodule formation. Several of these mutants showed a defect in MT46 expression in roots. This defect is either due to an inability to induce MT46 mRNA upon inoculation or due to a significant reduction in background levels of expression. Among these latter mutants is POODLE (*pdl*). The mutant poodle is characterized by a lack of macroscopic nodule primordia, and extensive cytological analysis failed to detect organized inner cortical cell divisions after rhizobial inoculation (Prabhu, 1998). In contrast to the absence of nodule morphogenesis, rhizobial infection of epidermal cells in poodle occurs at a frequency comparable to that observed in wild-type plants. However, all infections in POODLE arrest in the root epidermis or infrequently in the first cortical cell layer (Prabhu, 1998).

We have mapped the location of MT46 on the *M. truncatula* genetic map to approximately 2 cM from the site of the *pdl* mutation. A collection of 20 overlapping BAC clones of *M. truncatula* DNA form a contiguous region around the MT46a gene. End sequencing of the BAC clones revealed the presence of at least two additional apyrase genes. Therefore, this cluster contains at least 3 distinct, apyrase genes. The MT46b apyrase gene has not been precisely mapped but is apparently unlinked to the apyrase cluster containing MT46a. The presence of multiple apyrase genes in both soybean and *M. truncatula* (and likely other legumes) complicates the analysis of their role in nodulation. Our data clearly suggest that these genes are differentially expressed and we can expect that only one or a few of these genes will respond to rhizobial inoculation (e.g., GS52 and MT46a). The fact that these proteins are early nodulins suggests an important role in the nodulation process. However, further work is required to confirm their suggested role as Nod signal binding proteins.

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FUNCTIONAL CHARACTERIZATION OF A KRÜPPEL-LIKE ZINC FINGER GENE INDUCED DURING NODULE DEVELOPMENT

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Bacteria of the genera *Rhizobium, Azorhizobium, Bradyrhizobium,* and *Sinorhizobium* induce the development of nitrogen-fixing root nodules on leguminous plants. Symbiotic nodule development is controlled by signal exchanges between the two partners. Specific genetic programs are activated in both partners as demonstrated by the isolation of mutants affected in their symbiotic behaviour in both bacteria and different leguminous species (Schultze and Kondorosi, 1998). Using several differential screening and display strategies, genes coding for transcripts induced during nodulation, the so-called nodulin genes, have been identified, and their specific expression patterns at different stages of nodule development were determined. However, very few functional approaches have been tested for these genes and hence little is known about regulatory mechanisms controlling nodule organogenesis where they participate.

Using a cold-plaque screening procedure, we have previously identified transcripts induced during alfalfa (*M. saliva*) nodulation which exhibited sequence similarities to potential regulatory genes (Frugier et al. 1998). One of these, *Mscp17* (renamed *Mszpt2-1),* codes for a putative Krüppel-like (or TFIIIA) Cys_2/His_2 zinc finger protein. This large family of eucaryotic transcription factors plays central roles in various tissue-specific differentiation. Important members of this family are the Xenopus transcription factor IIIA (TFIIIA; Miller et al 1985), the Drosophila Krüppel and Hunchback early embryo regulatory genes (Stanojevic et al 1989). In plants, several TFIIIA zinc finger proteins have been described, although very little is known about their role in development. They are expressed predominantly in flowers although transcripts of certain members were detected in vegetative tissues such as roots. DNA binding activity has been demonstrated for some of the petunia EPF-type family proteins (Takatsuji 1998). However, only two plant TFIIIA genes, *Superman* in arabidopsis and *Indeterminate* in maize, have been functionally characterized, and both play roles in floral development (Sakai et al. 1995; Colasanti et al 1998).

Sequence analysis of the complete *Mszpt2-1* cDNA revealed that it belongs to the EPF-type family. This gene contains two canonical Cys_2/His_2 zinc finger motives, in contrast to *Superman* and *Indeterminate,* and a hydrophobic region in the C-terminal part (DLNL-box) conserved in most TFIIIA plant zinc fingers, likely involved in protein-protein interactions. In addition, a putative nuclear localization signal (NLS-box) is present in the N-terminal part. Thus, *Mszpt2-1* likely codes for a transcription factor, clearly divergent from the only plant Krüppel-like genes whose functions have been characterized. Southern blot analysis indicated that, in alfalfa, *Mszpt2-1* seems to be present in two copies or at least in two alleles, and as a single copy gene in *M. truncatula,* even though a related homologue might exist in both cases. Analysis of *Mszpt2-1* expression in different alfalfa organs showed basal expression levels in vegetative tissues but higher levels in flowers and nodules. Accumulation of the *Mszpt2-1* transcripts was also detected in nodules induced by different symbiotic bacterial mutants (Eps^-Kps^- and Bac^-), as well as in the adjacent root region. Thus,

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Mszpt2-1 induction in the nodule requires Nod factor signalling. *In situ* hybridization studies revealed that this expression is associated with vascular bundles from both roots and nodules.

In order to analyse potential functions of this Krüppel-like gene in plant organogenesis, we constructed *M. truncatula* lines expressing *Mszpt2-1* under the control of the strong constitutive 2x35S CaMV promoter, in sense or antisense orientation. After *in vitro* regeneration, the primary transformants of either sense or antisense plants were not disturbed in their growth characteristics. However, most of the *Mszpt2-1* sense overexpressing plants showed an abnormal flower phenotype, consisting in an unusually long style compared to the anther size, that became rapidly dehydrated, leading to inefficient pollinisation and to a strong fertility decrease. However, antisense plants did not exhibit this phenotype, suggesting that it may be due to interactions of the ectopically overexpressed *Mszpt2-1* protein with promoters that might be under regulation of homologues normally expressed in flowers, such as certain members of the petunia EPF-type family (Takatsuji et al 1994). Modulation assays were performed on the progeny of 8 genetically independent antisense T_0 lines, and plants originating from 3 of them developped only small white Fix$^-$ nodules even one month after infection with Sm41. Northern hybridizations performed with strand-specific probes demonstrate that the amount of sense *Mszpt2-1* transcripts in nodules and roots infected by *S. meliloti* was significantly reduced in the Fix$^-$ plants. Further study of this nodulation phenotype was carried out either using *S. meliloti* strains containing a constitutively expressed (□-ala) or a bacteroid-specific (nifH) lacZ fusion, and microscopic histological analysis. Bacterial infection as well as the early stages of nodule development took place normally in these plants, but no typical zone III was detected in mature nodules even though some infected cells were present in the apical region. However, the bacteria did not develop beyond the early stages of bacteroid differentiation, and did not display the typical radial pattern organized around a central vacuole, observed for the nitrogen-fixing symbiosomes in the central region. Moreover, a high number of amyloplasts was present in this tissue, indicating an absence of starch metabolization.

Altogether, these results showed that expression of the *Mszpt2-1* gene in antisense orientation affected nodule development by reducing bacterial invasion and preventing differentiation of the nitrogen-fixing zone III cells. Its function in a vegetative developmental process indicates that Krüppel-like zinc finger proteins play various roles in plant organogenesis.

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SUPPRESSION OF PLANT DEFENCE REACTIONS IN ALFALFA CELL CULTURES BY *SINORHIZOBIUM MELILOTI* SURFACE CARBOHYDRATES

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The soil bacterium *Sinorhizobium meliloti* is able to establish a symbiosis with its host plant alfalfa (*Medicago sativa)* as well as with the model legume *Medicago truncatula.* During the establishment of this symbiosis bacterial cells infect plant roots and induce a new organ, the root nodule. The bacterial *nod* genes, code for the synthesis and excretion of the nodulation factors (Nod factors), which are able to induce nodule organogenesis in the legume. The nodulation factors are structurally related to chitin, a common constituent of the fungal cell wall which is absent in higher plants. Chitin fragments are known to act as elicitors of defence reactions in cell cultures of various plant species. Because of the structural similarity between chitin elicitors and Nod factors we analysed whether these compounds may stimulate the plant defence system of *M. sativa.*

We first established elicitor-responsive cell suspension cultures of *Medicago sativa* (host plant) and *Nicotiana tabacum* (non-host plant) as a system to test the perception of biological active compounds (Baier et al, in press). Alfalfa suspension cultures respond to yeast elicitors with a strong alkalinization of the culture medium and a transient synthesis of activated oxygen species, generally termed oxidative burst. The alkalinization reaction as well as the oxidative burst were also observed when tobacco (*N. tabacum*) cell suspension cultures were treated with yeast elicitors. Dependent on the degree of polymerisation *N*-acetyl chitin oligomers induced the alkalinization response in both plant cell suspension cultures, while only tobacco cell cultures developed an oxidative burst. Suspension-cultured tobacco cells responded to *S. meliloti* nodulation factors with an alkalinization of 0.25 pH units and a significant oxidative burst. In contrast, addition of *S. meliloti* nodulation factors to suspension-cultured alfalfa cells induced no oxidative burst. Instead of an alkalinization, they showed a slight acidification of the culture medium. Apparently, alfalfa might have evolved a perception system that discriminates between nodulation factors and chitin oligosaccharides.

Beside the nodulation factors surface carbohydrates of *S. meliloti* play a curical role in the establishment of the symbiosis (Niehaus and Becker, 1998). Mutants of *S. meliloti* that failed to synthesize the acidic exopolysaccharide EPS I (succinoglycan) were unable to induce effective root nodules. In addition these strains induced a defence response on their host plants, probably preventing the infection by the symbiont (Niehaus et al., 1993). Further on, the bacterial lipopolysaccharide (LPS) plays a role in the interaction of *S. meliloti* with its different host plants. The specific *S. meliloti* LPS-mutant Rm6963 (Lagares et al. 1992), mutated in *lps*B, was shown to induce effective nodules on *M. sativa,* but failed to establish an effective symbiosis with *M. truncatula* (Niehaus et al. 1998). *M. truncatula* root nodules induced by Rm6963 also exhibited symptoms of plant defence reactions.

From these observations we propose that rhizobial surface carbohydrates act as suppressors of the plant defence system, enabling the symbiont *S. meliloti* to infect the host plant. Using the cell culture assay system, purified homologous and heterologous LPS were analysed for possible suppressor functions in alfalfa and tobacco plant suspension cultures. In alfalfa cell cultures the elicitor induced

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alkalinization and oxidative burst could be suppressed by the simultaneous application of purified *S. meliloti* LPS. In contrast *R. leguminosarum, Salmonella* or *E. coli* LPS showed no suppression of the elicitor induced response. None of the carbohydrate preparations were able to suppress the elicitor induced alkalinization and oxidative burst in the cell cultures of the non host plant tobacco.These data provide good evidence for a specific recognition of the *S. meliloti* LPS by the host plant which results in the suppression of the plant defence system, enabling the symbiont to establish an effective symbiosis.

In summary we were able to show that S. meliloti nodulation factors did not induce plant defence reaction in *M. sativa* cell suspension cultures. Additionally, *S. meliloti* LPS was able to suppress plant defence reactions induced by yeast elicitors. We suggest that *S. meliloti* LPS actively suppresses plant defence reactions in the host plant *M. sativa.*

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INVASION OF ALFALFA ROOT NODULES BY THE NITROGEN-FIXING BACTERIUM *RHIZOBIUM MELILOTI*

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In order for *R. meliloti* to invade the nodules it elicits on alfalfa, it must be able to synthesize at least one of the following three polysaccharides: succinoglycan, EPS II or a symbiotically active form of K antigen. A failure to produce one of these polysaccharides leads to the formation of immature nodules that are devoid of bacteroids and incapable of fixing nitrogen. Additional bacterial functions are required for *R. meliloti* to invade plant cells in the nodule and establish the intracellular state necessary for symbiosis.

Succinoglycan is an acidic polysaccharide composed of repeating octasaccharide subunits each containing seven β-linked glucose molecules, one β-linked galactose, and an average of one acetyl, one pyruvyl, and one succinyl modification (Aman et al 1981). Genetic and biochemical approaches have been used to gain an extensive understanding of succinoglycan biosynthesis. Taking advantage of the fact that the laundry whitener calcofluor fluoresces under UV light after binding succinoglycan, genetic screens for non-fluorescent mutants were used to isolate the genes involved in succinoglycan biosynthesis (Leigh 1985). Twenty-one of these genes, known as the *exo* and *exs* genes were found to lie in a 27 kb cluster on the symbiotic megaplasmid pSymB. An additional five *exo* genes have been identified in the genome (Niehaus and Becker 1998). A biochemical approach was then used to determine which of these genes were involved in each stage of succinoglycan subunit assembly. Briefly, radiolabeled precursors were added to permeablized cells containing different *exo* mutations, and the succinoglycan intermediate that accumulated was identified to determine the stage in the biosynthetic pathway for which the deficient gene product was needed (Reuber and Walker 1993).

R. meliloti synthesizes both high molecular weight (HMW) forms of succinoglycan consisting of thousands of octasaccharide repeating units, and low molecular weight (LMW) forms consisting of monomers, dimers and trimers of the octasaccharide repeating unit. These LMW forms of succinoglycan, especially the trimer, appear to be the species active in symbiosis. We have recently shown that very small amounts of succinoglycan trimer molecules co-inoculated on alfalfa with mutant strains unable to synthesize succinoglycan can rescue the symbiotic defect of these mutant strains (Wang et al 1999). There is evidence that *R. meliloti* produces LMW succinoglycan via two pathways: (i) a degradative pathway in which glycanases cleave HMW succinoglycan into LMW forms (York and Walker 1997) , and (ii) a direct synthesis pathway mediated by ExoP, ExoQ, and ExoT (Gonzalez et al 1998).

To gain insight into the nature of the requirement for succinoglycan, EPS II, or K antigen in nodule invasion, we have used fluorescence microscopy to examine the invasion process in *R. meliloti* strains that constitutively express green fluorescent protein (GFP). We have found that, while strains lacking active forms of all three polysaccharides are able to colonize curled root hairs, they are deficient in both initiation and elongation of infection threads. Furthermore, while strains producing active EPS II or K antigen were found to be proficient at forming infection threads, they did so less efficiently than strains producing active succinoglycan (B. Pellock, H.P.Cheng, G.C Walker, unpub). We are also interested in the regulation of succinoglycan biosynthesis. Several genes have been

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found to be required for proper regulation of succinoglycan biosynthesis, including *exoR, exoS, exoX, exsB, exoD* and *mucR* (Niehaus and Becker 1998). The *exoR95* and *exoS96* mutations have the most dramatic effects on succinoglycan synthesis. In both mutants, transcription of succinoglycan synthesis genes is increased leading to excessive succinoglycan production and very mucoid colonies (Doherty et al 1988). Interestingly, the *exoS96* mutant induces nitrogen-fixing nodules on alfalfa, but the *exoR95* mutant does not. Also, the *exoS* mutant but not the *exoR* mutant, produces less succinoglycan in the presence of ammonia (Doherty et al 1988).

We discovered that ExoS is the sensor in the ExoS/ChvI two-component regulatory system which has similarity to the ChvG/ChvI two-component system from *A. tumefacens* and is part of the EnvZ/OmpR family of two component regulatory systems. No homolog of known function has been found for ExoR, and its function is not yet known (Doherty et al 1988). We are now in the process of screening for additional regulators of succinoglycan synthesis (G.R.O. Campbell, G.C. Walker, unpublished).

R. meliloti also produces a strain-specific K antigen which can substitute for succinoglycan or EPS II if produced in an active form. Although three genetic regions required for the synthesis of K antigen have been sequenced and characterized (Kiss et al 1997, Williams et al 1990a, Kereszt et al 1998), none of these loci contained glycosyl transferases as have been found in succinoglycan and EPS II biosynthetic pathways. This suggested that there must be additional genetic loci involved in K antigen synthesis. To isolate additional loci involved in the synthesis of K antigen, we screened ca. 100,000 mutagenized colonies for resistance to bacteriophage 16-3, which requires the K antigen to infect *R. meliloti.* Through this method we were able to isolate four new classes of K antigen mutants from at least three different complementation groups (Campbell et al 1998). Interestingly, two of these classes also have defects in LPS production. One of these genetic loci, *rkpK* encodes a UDP-glucose dehydrogenase (Kereszt et al 1998).

The *bacA* gene product is needed at a later step in nodule development. The *bacA* gene encodes a predicted membrane transporter, and *bacA* mutants senesce at an intermediate time point in symbiotic development when wildtype bacteria would normally begin to differentiate into nitrogen fixing bacteroids(Glazebrook et al 1993). We have found *bacA* homologs in the pathogenic bacteria *Brucella abortus* and *Salmonella typhimurium* (K. LeVier, A. Ichige, G.C.Walker, unpublished). In *B. abortus, bacA* mutants had decreased survival levels in murine macrophages *in vitro,* and greatly attenuated pathogenicity in mice. These proteins may be involved in alerting bacteria to their host environments, promoting cellular changes that allow survival within eukaryotic cells.

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STRUCTURE AND DEVELOPMENT OF INFECTION THREADS

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In pea and other temperate legumes, *Rhizobium* gains entry to host root tissues through tip-growing infection threads. These are tubular ingrowths of the plant cell wall which originate from curled root hair cells. Having traversed the epidermis, the bacteria are once again released into the extracellular matrix. Infection is propagated because transcellular threads are reinitiated in root cortical cells and subsequently in the invasion zone of the developing nodule Here, we discuss some of the plant components that might be involved in the colonisation of the extracellular matrix.

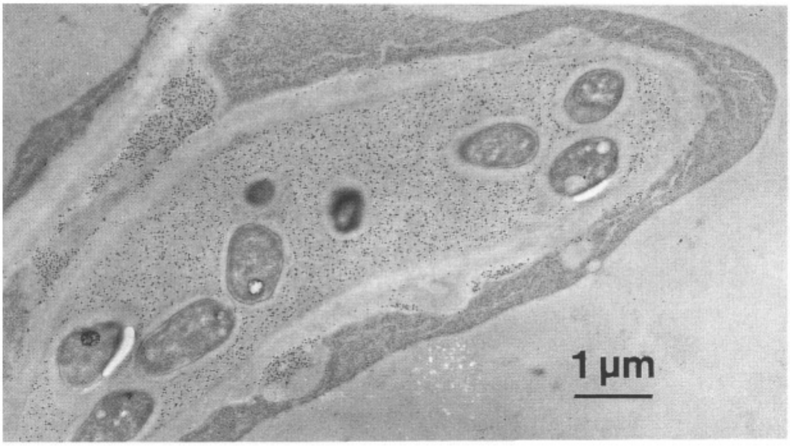

Figure 1.

Bacteria within infection threads are normally surrounded by an extracellular matrix, as indicated in Fig. 1. Using a monoclonal antibody, MAC265, a 110kDa plant glycoprotein was localised to the infection thread lumen. In the immunogold labelled micrograph, the higher density of colloidal gold particles at the margins of the thread wall indicate that targetted secretion of matrix glycoprotein (MGP) could be a factor contributing to the growth of the infection thread. MAC265 antigen was also found in intercellular spaces, indicating that the thread lumen resembles the extra-cellular matrix (VandenBosch et al. 1989; Rae et al. 1992). MGP is also released from root tips.

MAC265 recognises a carbohydrate epitope on MGP. The glycoprotein was extracted from root exudate and concentrated by immunoaffinity chromatography using protein G sepharose coupled to MAC265. Immunopurified glycoprotein had a blocked N-terminus and was resistant to cleavage by many proteases. Fourier-transform infra-red spectroscopy indicated a high carbohydrate content (>60%). Following treatment with chymotrypsin, a 40 kDa polypeptide was isolated by gel electrophoresis. This fragment, which retained reactivity with MAC265, yielded 29 amino-acids by N-terminal sequencing (underlined in Fig. 2). A 650 base-pair cDNA clone corresponding to MGP was obtained by 3'RACE PCR using mRNA isolated from *Rhizobium*-inoculated pea roots. The DNA sequence indicated that MGP-ER2 encodes an extensin-like glycoprotein with (hydroxy)proline-rich domains. Interestingly, the sequence included four repeats of the motif SP_5, whereas SP_3 and SP_4 are more common in the extensins of higher plants.

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MGP-ER2	**VfNDS-E**	**MtN12**
....	....	VYH**SPPPP**
TPHKKPYKYP**SPPPPP**	.......HYHYS**SPPP**	VHHTYPKPVYH**SPPPP**
VHTYPHPHPVYH**SPPPP**	VHTYPHPHPFYH**SPPPPP**	VHTYPHPKPVYH**SPPPP**
HKKPYKYS**SPPPPP**	TPHKKPYKYP**SPPPPP**	VHTYPKPVYH**SPPPP**
VHTYPHPHPVYH**SPPPPP**	VHTYPHPHPVYH**SPPPPP**	VHTYVPHPKPVYH**SPPPP**
TPHKKPYKYP**SPPPPP**	TPHKKPYKYP**SPPPPP**	....
AHTYPPHVPTPVYH**SPPPP**	VHTYPPHVPHPVYH**SPPPP**	**VHTYPPHVPHPVYHSPPPP**
VY**SPPPP**	VY**SPPPP**	VH**SPPPP**
AYYYK**SPPPP**	HYYYK**SPPPP**	HYYYK**SPPPP**
YHH*	YHH*	YHN*

Figure 2 (above) shows very high homology between the MGP sequence from pea and the deduced amino acid sequence for VfNDS-E, a nodule-enhanced extensin from *Vicia faba*, previously isolated as cDNA in a subtractive hybridisation study (Perlick, Puhler 1993). Similarly, MtN12 from *Medicago truncatula* was obtained from a nodule-enriched cDNA library (Gamas et al. 1996). The motif "VHTYPHPHPVYHSPPPP" is particularly strongly conserved. However, neither of these studies involving cDNA cloning was able to identify the gene product as a component of the infection thread matrix because no antibody was available for immunolocalisation studies.

Exploiting the fact that MGP is secreted from pea root tips, we have developed an *in vitro* bioassay for its insolubilisation by examining factors that reduce its secretion into the surrounding medium. We found that MGP can easily be insolubilised in a time-dependent manner by a process that is dependent on H_2O_2. Known inhibitors of peroxidase (e.g. salicyl hydroxamate) increase the rate of MGP release from root tips. We therefore suggest that peroxide-induced hardening of MGP could influence both the initiation and the propagation of the infection thread.

Modifying the physical properties of the extracellular matrix could have several important consequences during the infection process. (1) In the curled root hair, solidification of the gum surrounding attached rhizobial cells could create a plug which would allow growing and dividing bacteria trapped on the inside to generate an inward thrust which could initiate infection thread development from the kink in the curled root hair. (2) In the lumen of the infection thread, hardening of the matrix material could explain why bacterial growth is only observed at the tip (Gage et al. 1998) where, presumably, the matrix is still fluid. (3) Finally, it is easy to imagine how the abortion of infection threads might be due to premature hardening of the luminal matrix in the region of the growing tip.

Possible sources of peroxide in the extracellular matrix could include NAPH oxidase, diamine oxidase or oxalate oxidase. The regulation of these activities could be very important in symbiosis.

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UNUSUAL LPS STRUCTURAL FEATURES AND SYMBIOTIC FUNCTION

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1. Introduction

Rhizobial lipopolysaccharides (LPSs) are the principal molecules that comprise the outer leaflet of the outer membrane of these gram-negative soil bacteria, and are essential for establishing nitrogen-fixing symbioses with legume host plants (see the recent review by Kannenberg et al. 1998). The LPSs are complex molecules consisting of a polysaccharide (O-chain) that is attached to an oligosaccharide (core) which, in turn, is attached to a lipid (lipid A). The LPSs from rhizobial mutants which lack the O-chain, contain insufficient levels of normal O-chain, or which contain only truncated O-chain are defective in symbiotic infection. The LPS from certain species of rhizobia have very unusual structural features compared to those from enteric bacteria. This paper describes these unusual structural features, their biosynthetic origins, and their possible function(s) in symbiotic infection.

2. Material and Methods

2.1. Bacterial Strains.

Bacterial strains were *R. leguminosarum* (*Rl*) bv. viciae, and *R. etli* (*Re*).

2.2. LPS Isolation and Characterization.

LPSs were isolated by hot phenol water extraction and purified by gel-filtration chromatography. The carbohydrate and lipid A portions were separated by mild acid hydrolysis and organic extraction. Samples were analyzed by determining the glycosyl and fatty acyl residue composition, by mass spectrometry, in the case of the lipid A, after de-O-acylation, by polyacrylamide gel electrophoresis in the presence of deoxycholate, and by nuclear magnetic resonance spectroscopy. The methods for LPS purification and characterization have been summarized in a recent review (Kannenberg et al. 1998).

3. Results and Discussion

The LPSs from *Rl* and *Re* strains have unique structural features when compared with LPSs from enteric bacteria (see recent review by Kannenberg et al. 1998). The O-chains from both types of bacteria often contain deoxyhexosyl residues, however, those from *Rl/Re* often, but not always, contain highly methylated glycosyl residues. The rhizobial core oligosaccharide is very different from that found in enteric LPSs. The core from *Rl/Re* LPS is common to all strains of these species, contains galacturonic acid (GalA), has an external 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), and is completely devoid of heptose, a common component of the inner core region of enteric LPSs. The *Rl/Re* lipid-A is unique in that it is devoid of phosphate, contains GalA at the 4' position, and 2-aminogluconate replaces the glucosamine-1-phosphate normally found in enteric LPSs. In addition the *Rl/Re* lipid-A contains only hydroxy fatty acids, one of which is the very long chain 27-hydroxyoctacosanoic acid (27-OHC28:0). Fatty acid analysis of the LPS from numerous rhizobial species has shown that this long chain fatty acid is a common LPS feature of the *Rhizobiaceae* (Bhat et al. 1991).

The biosynthetic basis for the absence of phosphate in *Rl/Re* LPSs has been shown to be due to the presence of 4'- and 1-phosphatases that are unique to these rhizobial species (see Kannenberg et al. 1998 review). These enzymes can remove both phosphates from a precursor that is common to both *Rl/Re* and *E. coli* LPS, $\text{Kdo}_2\text{Lipid-IV}_A$. The 27-OHC28:0 fatty acyl residue can be added to

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Kdo$_2$Lipid-IVA using a unique acyl carrier protein (ACP-XL) and, presumably, acyl transferase found in *Rl* and *Re* (see Kannenberg et al. 1998 review). However, the galacturonosyl transferase activities which would be required for addition of GalA to the lipid-A and core regions have not been reported. A spontaneous mutant of *Rl*, 24AR, which does not have the 4'-phosphatase activity has an LPS that contains both the 4'- and 1-phosphate groups, but does not have any detectable levels of Gal in either the core or lipid-A regions. This would suggest that removal of the phosphate is necessary prior to GalA addition. The 24AR mutant still contains a number of the other unique *Rl* enzyme activities, such as the mannosyl transferase which adds Man to the core region, the ACP-XL/acyltranferase system, etc.

The level of O-chain that is present on the bacterial surface can be dictated by the structure of the lipid-A and/or core region. Since the length and amount of O-chain is a requirement for infection, the regulation of lipid-A-core synthesis would also regulate whether or not the symbiont bacterium invades the host cell, and perhaps also determine if changes in structure can occur that are important for infection and bacteroid development.

Recently, we (see abstract by Kannenberg) have found that the LPS from nodule *Rl* bacteria, or from bacteria grown at low pH or low O_2, are extracted into the phenol layer during hot phenol/water extraction rather than largely into the water layer as is found for the LPS from a laboratory culture. Thus, the LPS becomes more hydrophobic during symbiosis. Since the core region of *Rl* LPS is highly acidic while the O-chain is relatively hydrophobic due to deoxy- and methylated glycosyl residues, the increased amounts and/or length of O-chain could increase the LPS hydrophobicity. In addition, increases in the level and chain length of the fatty acyl residues would also increase LPS hydrophobicity. The chemical analysis of the hydrophobic LPS from is discussed in the abstract by Kannenberg. Interestingly, in addition to this increase in LPS hydrophobicity, Kannenberg (see abstract) found that the hydrophobicity of the entire bacterium increases during symbiosis. Thus, the relatively highly acidic nature of the surface of normally cultured bacteria due to acidic EPS, CPS and the LPS core region, must be dramatically altered during symbiotic infection. The function of this increase in hydrophobicity may be necessary for the invasion of host cells which occurs by an endocytotic process. One of the lipid-A changes that occurs during infection appears to be an increase in the level of 27OHC28:0. Of course this change would contribute to the increase in hydrophobicity. This long-chain fatty acid can span the entire membrane lipid bilayer and, therefore, could also be important in increasing the stability of the bacterium once it is inside the host cell. These changes in the LPS may also important for the exchange of metabolites between the bacterial and host symbionts.

4. Acknowledgements

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TWO *BRADYRHIZOBIUM JAPONICUM* GENES ENCODING PUTATIVE SENSOR PROTEINS

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1. Introduction

The potential to perceive changing environmental conditions and to specifically react on this new information is essential for all organisms. In any bacterial species, the first steps of signal transduction are mediated by a number of protein pairs, consisting of a sensor (protein histidine kinase) and a regulator (transcriptional activator or repressor). In the *Rhizobiaceae*, quite a number of sensor/regulator pairs have been described. As the transition from the free-living state to the symbiotic form means profound changes of the environmental conditions, these mediators of signal transduction are assumed to be involved in the competitiveness of nodule formation, host specificity, bacterial infection, regulation of oxygen-sensitive gene expression such as nitrogen fixation and carbon source metabolism.

2. ElmS and ElmR, a novel sensor/regulator pair of *B. japonicum*

Extended DNA sequencing of *B. japonicum* genomic fragments next to the *sipS* gene (symbiosis specific signal peptidase, Müller et al 1995) lead to the identification of a genetic locus encoding a putative sensor/regulator pair, designated *elmS* and *elmR*. The alignment of the deduced amino acid sequences (469 aa and 214 aa) exhibits good agreement with numerous other two component systems. Amino acid positions known to be essential for function are perfectly conserved.

The C-terminal residues of ElmS represent highly conserved domains of histidine protein kinases (HPK). Unlike most other members of this protein family, the distance between conserved motives DXGXG and GXG is only 7 amino acid residues. This trait is shared with a small subgroup of HPKs, such as MxcQ of *M. organophilum XX* (Xu et al 1995) and MxaY of *P. denitrificans* (Harms et al 1993). As the molecular masses of the three proteins are similar and the periplasmic domains (defined by two transmembrane segments in each case) also share significant sequence similarities it is reasonable to assume that similar signals might be recognized by the three proteins, since the periplasmic domains are involved in signal perception. MxcQ has been shown to be involved in the regulation of methanol dehydrogenase (MDH) in *M. organophilum XX,* and formate has been identified as the signal. The structural relatedness of the putative transcriptional regulator protein ElmR to MxcE of *M. organophilum XX and* MxaB of *P. denitrificans* further substanciates this hypothesis. Unlike most other examples of sensor/regulator pairs, ElmS and ElmR are organized in two adjacent but opposite operons. Their start codons are separated by only 84 nucleotides.

Mutants carrying a truncated *elmS* gene, did not exhibit an altered phenotype in standard free-living conditions or in symbiosis with soybeans. Experiments to test these mutants in different growth conditions and to construct and assay *elmR* mutants are in progress. The resulting mutants will be tested on different host plant species.

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3. A mutation in *bdfA*, encoding another putative sensor protein affects the symbiotic interaction

Sequence analysis of a second DNA region inserted by Tn*5* in mutant 3652, originally described by Regensburger et al (1986), revealed a gene of another putative sensor protein (873 amino acids), which was designated *bdfA*. Unlike other prokaryotic sensor proteins, the phosphate accepting histidine site is replaced by a tyrosine, and in the close vicinity there is a conserved tyrosine sequence motif which is typical for eukaryotic kinases. The gene for the corresponding regulator is not located in the vicinity of *bdfA*. DNA sequence data suggest that *bdfA* is organized in a common operon with *dfp*, *dut* (upstream) and at least an additional ORF6 (downstream). This is confirmed by insertion mutant I-105-08, disrupting the transcription of the operon. As a result, I-105-8 exhibits a severe symbiotic phenotype: the bacteria induce the formation of root nodules but almost completely fail to be released from the infection threads. This phenotype is more severe than that of the original Tn*5* insertion in mutant 3652 which is characterized by a reduced symbiotic nitrogen fixation rate.

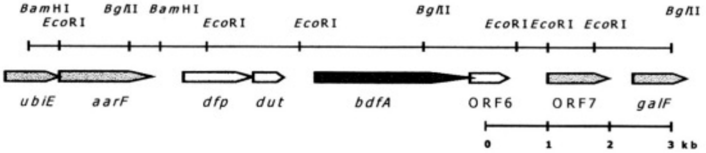

Fig. 1: restriction and genetic maps of the *bdfA* region

dut encodes the deoxyuridine triphosphatase (dUTPase), an essential enzyme in the biosynthesis of deoxynucleotides. Based on experiments in *E. coli*, the reduction of ribonucleotides to deoxyribonucleotides, catalyzed by ribonucleotide reductase (RNR), appears to depend on FMN-Drp. In other microorganisms the existence of several classes of RNRs has been shown, which are required in either aerobic or anaerobic conditions (Jordan A, Reichard P 1998). Because a *bdfA* mutant exhibits only a symbiotic phenotype it is hypothesized that the *B. japonicum* *dfp/dut/bdfA/*ORF6 operon is expressed in the microaerobic bacteroid state to activate specific RNRs which are required to form uracil. This is a key substrate for the formation of **β-alanine** which is required for the synthesis of pantothenate, the precursor molecule of the essential compound coenzyme A. ORF6, which overlaps with *bdfA*, encodes a putative inner membrane protein which carries a highly conserved nucleotide binding site. It is tempting to speculate that this gene product binds a nucleotide, thereby sensing the availability of dNTPs which are required as energy sources and for DNA synthesis.

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DIFFERENTIAL EXPRESSION PATTERN OF TWO APYRASE CDNAS EXPRESSED IN THE ROOTS OF SOYBEAN (*GLYCINE SOJA*).

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Recently, Etzler *et al.* (1999) reported the isolation of a lectin (e.g., DB46) from roots of the legume *Dolichos biflorus.* This protein was shown to bind the lipo-chitin Nod signals produced by two rhizobial symbionts of *D. biflorus*, *B. japonicum* USDA110 and *Rhizobium* sp. NGR234. In addition, DB46 was reported to possess a non-specific NTPase activity (i.e., apyrase) that was significantly enhanced upon Nod signal binding. Thus, DB46 is a possible candidate for a Nod signal receptor that, in a bound state, could activate cellular processes by way of its apyrase activity.

We isolated two soybean cDNAs, termed GS50 and GS52, which encode apyrase proteins of 50 kDa and 52 kDa, respectively. Northern analyses indicate that GS52 mRNA is expressed in roots, hypocotyls and flowers, whereas GS50 mRNA was expressed weakly and only in roots and flowers. Western blot analysis using antigen-purified antibodies to both GS50 and GS52 showed similar patterns of tissue localization.

GS50 was expressed constitutively in roots at a relatively low level under all tested conditions. However, GS52 mRNA levels in roots were significantly increased within six hours after inoculation with *Bradyrhizobium japonicum.* Therefore, GS52 can be classified as an early nodulin. Preliminary mRNA expression data also suggest that GS52 is repressed in the presence of 20 mM nitrate, whereas expression of GS50 was not affected.

The differential expression pattern of GS50 and GS52 suggests that they likely have distinct cellular functions. The rapid induction of GS52 expression upon inoculation and the repression by nitrate suggest that this protein may have a function in the nodulation process.

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Acknowledgements

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USE OF PEA (*PISUM SATIVUM L.*) MUTANTS IMPAIRED IN NODULATION AND ROOT FORMATION TO STUDY THE ROLE OF PHYTOHORMONES IN NODULE DEVELOPMENT

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Nodule formation as a differentiation process depends on plant endogenous hormonal status. Two approaches to investigate the functions of phytohormones in the legume-rhizobial symbiosis have been proposed.

First, the features reflecting the plant endogenous hormonal status (sensitivity to exogenous auxin and cytokinine and content of hormones) of Nod^{-} (E2 (*sym5*), R25 (*sym8*)) and Nod^{++} (RisFixC, nod3) pea mutants kindly provided by T. LaRue, K. Engvild and E. Jacobsen, were characterised. The dependance between supernodulation phenotype and auxin status was revealed: both Nod^{++} mutants were more sensitive to exogenous auxin on the stem and root levels and for RisFixC the increased content of endogenous IAA in aseptic seedlings and during rhizobia inoculation was shown. Nod^{-} line E2 also was more sensitive to auxin than Nod^{-} line R25 and their parental cv. Sparkle. And all mutants do not differ from their parents on sensitivity to exogenous cytokinin. Our results are in good agreement with the mutants nature. Nod^{++} forms have a broken autoregulational control of nodule formation, which is proposed to be phytohormonal one (Gresshoff, Caetano-Anolles, 1992). Symbiosis of mutant line R25 is blocked on the early stage of symbiotic relations with rhizobia – bacteria binding to root hairs (Kneen, LaRue, 1986). This stage is independent of plant hormones and so mutations in *sym8* gene have not to influence the plant hormonal status. Line E2 has a violation on the stage of nodule meristem formation (Kneen, LaRue, 1988), which depends on phytohormones. And earlier it was shown that plants of line E2 can develop nodules under the low temperature (12°C) (Fearn, LaRue, 1991a) or processing by inhibitors of ethylene action (Fearn, LaRue, 1991b). So mutations in *sym5* gene lead to the phytohormonal system disturbances.

Second, the symbiotic ability of pea hormonal mutant SGE*crt* (curly root) in newly identified gene *crt* controlling root formation was studied. This mutant has been shown to and double increased content of free auxin (IAA) and decreased in ten times nodules number.

Thus we can conclude that:

1. violationes on some nodule formation stages are connected with the changes in plant hormonal system.
2. pea hormonal mutants may be the nodule forming mutants too.

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RHIZOBIUM BACTEROIDS EXPRESS HYDROPHOBIC LIPOPOLYSACCHARIDES

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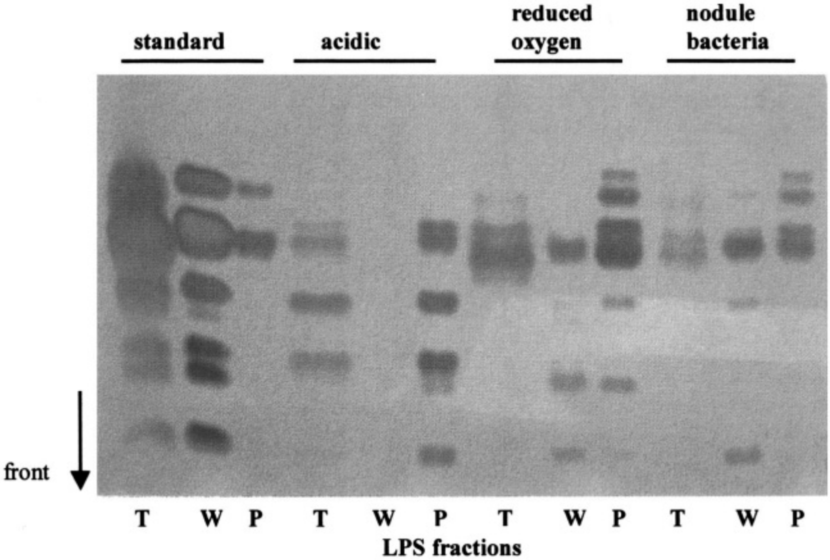
Rhizobium lipopolysaccharides (LPSs) are essential for symbiotic bacteroid development in pea nodules. Different LPSs are expressed in free-living bacteria and bacteroids (see Figure). LPSs from rhizobia cultured at conditions considered to mimic nodule physiology (mildly acidic, or reduced oxygen) were chemically characterized and compared to LPSs from standard-grown and nodule bacteria: LPSs from acidic- and reduced oxygen-grown cells were more hydrophobic and extracted exclusively, or largely into phenol during phenol/water extraction. Compared to their hydrophilic counterparts, hydrophobic LPSs contained (i) higher proportions of long chain fatty acids attached to their lipid A moieties, (ii) larger amounts of neutral sugars and reduced amounts of charged sugars in their O-chain moieties, as well as (iii) higher degrees of acetylation or methylation. Overall, LPSs from low oxygen cultured rhizobia resembled those of nodule bacteria indicating that, during bacteroid development, a switch takes place from hydrophilic to hydrophobic LPSs.



Gel banding patterns of LPSs isolated *from R. leguminosarum* 3841 cultured at different growth conditions and compared with LPSs from nodule bacteria. Bacteria were cultured as indicated. Total LPS fractions (**T**) extracted into sample buffer before fractionation. LPSs were then separated into water-soluble (**W**) and phenol-soluble (**P**) fractions. LPS fractions were separated by electrophoresis on polyacrylamide gels (18%), followed by a carbohydrate-specific silver-staining method.

CHARACTERIZATION OF A CHROMOSOMAL NODI HOMOLOG

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NodI and NodJ are two proteins that belong to the ABC-transporters. Their corresponding genes are located in the same operon as nodABC. The products of these genes are able to synthesize a core lipochitin oligosaccharide (LCO). Therefore NodI and NodJ were expected to be the transporters of LCO's and in the past it has indeed been shown that they are involved in this transport (Spaink et al. 1995). Mutations in nodI or nodJ did slow down secretion, however they did not abolish it completely. The goal of this study was to look for homologous genes on the chromosome of rhizobia, of which the products could secrete LCO's.

A PCR on chromosomal DNA isolated from three strains of rhizobia was performed with degenerate primers based on the ATP-binding regions. Fragments of 400 bp were cloned in the pGEM-T vector (Promega, Wisconsin USA) and sequenced. This yielded 7 interesting clones. Three clones, derived from two different strains, were almost completely identical and are the most homologous to NodI. One of these clones was used for further identification. This fragment was used to screen a cosmid library from Rhizobium leguminosarum bv. trifolii strain LPR5045. 11 kb Of DNA, containing the complete transporter (hereafter called hndI and hndJ), was obtained and sequenced to completion. To investigate if HndI and HndJ were involved in LCO transport and nodulation we created mutants by gene replacement and transduction in strains LPR5045 (cured) and RBL5523 (wild type), resulting in strains RBL5972 and RBL5971 respectively. Several plasmids containing nod genes were crossed to RBL5972 and used in transport assays and plant inoculation experiments.

TLC analysis of LCO's isolated from the mutants at different time points showed the same as with the cured strain containing LCO synthesis genes, that is that they are able to transport LCO's even in the absence of nodI and nodJ and in the same time scedule. Also the plant experiments didn't show differences in nodule numbers and nodulation time. In fact, RBL5972 forms somewhat more normal looking nodules than RBL5045. It has to be seen if this is dependent on the plant species used. In our experiments we used Vicia sativa because of the sym plasmid present in our Rhizobium strain. The genetic background of this strain is Rhizobium leguminosarum bv. trifolii, so it could be interesting to repeat these experiments on Trifolium, with mutants containing the right sym plasmid. It also indicates that each transport system has some substrate specificity.

In contrast, strains overproducing HndI and HndJ do not show LCO synthesis at all. Silica TLC analysis shows an extra spot appearing under the LCO spots, so a more hydrophilic compound is synthesized. This compound seems to be resistant to chitinase, although the spot diminishes after 180 minutes and even more when chitinase is added in the nodJ mutant overproducing HndI and HndJ. At this timepoint the strain seems to start LCO synthesis. This compound could be an intermediate of two different metabolic pathways, one leading to LCO's and the other leading to something unknown. This compound would than be secreted before it can be used for LCO synthesis. Another possibility could be that the overproduced transport system uses so much ATP that it creates a shortage for other ATP using proteins. Isolation and analysis of this compound could be helpful to elucidate this problem.

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GLYCOSYL SEQUENCE AND UNIQUE FEATURES OF THE O-POLYSACCHARIDE FROM THE *RHIZOBIUM ETLI* CE3 LIPOPOLYSACCHARIDE

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The O-antigenic polysaccharide of the *Rhizobium etli* strain CE3 LPS was isolated and structurally characterized using chemical degradations in combination with GLC-MS, electrospray-ionization mass spectrometry, MS/MS, MALDI-TOF-MS, and ^{1}H, ^{13}C, and ^{1}H COSY and TOCSY NMR analyses of the native polysaccharide and the derived oligosaccharides. Chemical degradations included the Smith degradation, lithium-catalyzed degradation of uronic acid residues, and base-catalyzed beta-elimination of the uronosyl residues. The CE3 O-chain polysaccharide was found to be a unique, relatively low MW glycan having a fairly discrete size, with surprisingly little variation in the number of repeating units (DP = 5). The polysaccharide is highly *O*-acetylated and contains a variety of *O*-methylated glycosyl residues, rendering the native glycan somewhat hydrophobic. The MW of the major de-*O*-acetylated species, including the reducing end Kdo residue, is 3330 Da.

The polysaccharide contains a trisaccharide repeating unit having the structure: **→4)-α-D-Glc*p*A-(1 →4)-[α-L-3-*O*-Me-6-deoxyTal*p*-(1→3)]-α-L-Fuc*p*-(1→**. Its non-reducing end terminates with a capping sequence of **α-L-2,3,4-tri-*O*-Me-Fuc*p*** attached to O-4 of a GlcA*p* residue. The reducing end has the sequence: **-3)-D-Man*p*-(1→3)-L-QuiNAc*p*-(1→4)-α-Kdo,** where QuiNAc represents *N*-acetylquinovosamine (2-*N*-acetamido-2,6-dideoxyglucose). The reducing-end Kdo residue links the O-chain polysaccharide to the core region oligosaccharide, resulting in a unique location for a Kdo residue in LPS, removed four residues distally from the lipid A moiety (Forsberg, Carlson, 1998). The 3-linked QuiNAc residue, which is not part of the repeating unit, is uncommon and originally believed required for efficient O-chain attachment to the LPS core region. But the *R. etli* mutant CE166 containing the PCOS 109.11 plasmid insertion lacks QuiNAc yet is able to synthesize LPS with substantial O-chain. The structure of the O-chain and core region in this mutant is under study.

Structural heterogeneity in the O-chain arises mainly from *O*-acetyl and *O*-methyl substitution. Linkage analysis using trideuteriomethyl iodide indicates that a portion of the 2,3,4-tri-*O*-methylfucosyl capping residues, typically 15%, are replaced with 2-*O*-methyl- and/or 2,3-di-O-methylfucose residues; the percentage varies and is apparently affected by culture conditions. In addition, approximately 25% of the 3,4-linked branching fucosyl residues and 10% of the 3-linked fucosyl residues are 2-*O*-methylated. This indicates that almost all of the 2-O-methyl fucose present in the glycan derives from the branching, 3,4-linked residues. Deuterium incorporation further indicates that there is essentially no variability in the methylation of the terminal 3-*O*-Me-6-deoxytalose residues (e.g. 2-0-Me or 2,3-di-*O*-Me, etc., 6-deoxytalose residues were not detected). The unique structures of the *R. etli* LPS core region [Forsberg, Carlson 1998], and lipid A moiety [Bhat et al 1994], were published previously. The present results raise interesting questions on the role of O-chain structure during the infection process.

Forsberg LS, Carlson RW (1998) J. Biol. Chem., 273, 2747-2757.
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INVOLVEMENT OF THE SECRETED EXPE1 PROTEIN AND ITS TRANSPORT SYSTEM IN THE BIOSYNTHESIS OF GALACTOGLUCAN BY *Sinorhizobium meliloti*

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Bacterial exopolysaccharides (EPS) are essential for establishment of the nitrogen-fixing symbiosis between *Sinorhizobium meliloti* and the host plant *Medicago sativa.* Under standard conditions *S. meliloti* secretes an acidic EPS termed succinoglycan (EPS I). This EPS is composed of octasaccharide repeating units containing one galactose and seven glucose residues and can be decorated by acetyl, succinyl and pyruvyl groups. In addition, *S. meliloti* also have the ability to produce a second EPS, termed galactoglucan (EPS II), which can replace EPS I in nodule invasion. EPS II is composed of alternating glucose and galactose residues which are decorated by acetate and pyruvate. The production of EPS II was observed in the presence of a mutation in the regulatory genes *exp*R or *muc*R; phosphate-limiting conditions; extra copies of the *exp* gene cluster responsible for EPS II biosynthesis or extra copies of the transcriptional regulator *exp*G.

The sequence of the *exp* gene cluster directing the biosynthesis of EPS II is known and the inferred properties of the encoded gene products implied that these proteins are involved in regulation of *exp* gene expression, diphospho-sugar biosynthesis, export and polymerization (Becker et al. 1997). Homologies of the deduced amino acid sequences from ExpD1 and ExpD2 to components of ABC protein secretion complexes implied that the N-terminal domain of the ExpD1 protein represents the membrane-spanning domain of ABC transporters and the C-terminal portion the ATP-binding cassette (ABC) domain. ExpD2 displays homologies to accessory factors of these secretion systems. The protein encoded by *exp*E1 located immediately downstream of the *exp*D1-*exp*D2 operon was found to be homologous to the *R. leguminosarum* extracellular protein NodO involved in nodulation (Becker et al. 1997). Results showed that ExpE1 is able to bind Ca^{2+} in accordance with its predicted 15 repeats of the nine residue GGXGXDXUX Ca^{2+} binding motif.

By immunodetection ExpE1 was detected in the cytoplasm and in the growth medium of *S. meliloti.* This result is in accordance with similarities of ExpE1 to secreted proteins like toxins, metalloproteases or NodO. ExpE1 was not detected in the growth medium of non-polar *exp*D1 and *exp*D2 deletion mutants. Since ExpD1 and ExpD2 are homologous to ABC transporter and membrane fusion proteins, respectively, of type I secretion systems, these results indicate that ExpE1 is secreted by a type I secretion system involving ExpD1 and ExpD2.

A non-polar *exp*E1 deletion mutant that carried additional mutations in *exo*Y and *muc*R, grown under phosphate-limiting conditions, lacked detectable extracellular HMW or LMW-EPS II. This indicates that ExpE1 is either directly involved in a biosynthetic step or in the secretion of EPS II. Since supplying ExpE1 *in trans* did not restore the ability of the *S. meliloti exp*E1 deletion mutant to produce extracellular EPS II, the extracellular form of ExpE1 seems to be not functional in the synthesis or secretion of EPS II. This implies that to fulfill its function ExpE1 has to be synthesized and secreted by the cell that synthesizes EPS II. In the culture supernatants of mutants in *exp*D1 and *exp*D2 no EPS II was detected indicating that ExpD1 and ExpD2 can be directly or indirectly required for EPS II synthesis or secretion. Since secretion of ExpE1 required ExpD1 and ExpD2 and mutants in *exp*E1 lacked detectable extracellular EPS II, it seems that ExpD1 and ExpD2 are indirectly required for EPS II production.

Analysis of the symbiotic properties of *exp*D1, *exp*D2 and *exp*E1 mutants revealed that these genes are not required for symbiotic nitrogen fixation and that nodulation was not affected.

Becker A et al (1997) J Bacteriol 179,1375-1384.

RHIZOBIUM SP. BR816 PRODUCES A MIXTURE OF CLASSICAL NOD FACTORS AND NOVEL NOD FACTOR LIKE STRUCTURES WITH A *N*-ACETYL GLUCOSAMINITOL AS THE REDUCING SUGAR

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An efficient symbiosis between rhizobia and leguminous plants involves a multistep reciprocal signal-exchange process. The secreted lipochitooligosaccharides or Nod factors (NFs) are the main determinants of host specificity. With their specific decorations, NFs trigger cortical cell division only in compatible legume plants, leading to nodule formation. The genes required for synthesis and transport of NFs are the nodulation (*nod*) genes. The broad host range strain, *Rhizobium* sp. BR816, isolated from the nodules of the tropical tree *Leucaena leucocephala* nodulates bean (*Phaseolus vulgaris*) and other tropical leguminous plants (Hernández-Lucas et al., 1995). Our research group identified and characterised the different regulatory and structural nodulation genes of BR816. To elucidation its host-specific determinants, we determined the NF structures of this strain.

LCOs were purified from 6-liter apigenin-induced cultures of *Rhizobium* sp. BR816 by using amberlite XAD-4. Adsorbed components were eluted with methanol, dried and further purified on an open C-18 reversed-phase column. After elution with 50% acetonitrile, the components were separated by C-18 reversed-phase HPLC with a gradient of 20% aqueous acetonitrile to 100% acetonitrile in 50 minutes. Fractions of interest were extracted again with butanol. The mass spectra obtained with FAB$^+$-MS and FAB$^-$MS, together with additional chemical analysis, showed at least three families of Nod factors. Each family consists of members separated by 2 mass units [$(M+H)^+$ ions: 1393-1395-1397, 1435-1437-1439, 1477-1479-1481]. All are pentamers with common C18:1 (*m/z* 1393, 1435 and 1477) or C18:0 fatty acids (other *m/z* ions). They are all *N*-methylated and C-6 carbamoylated at the non-reducing end and C-6 sulfated at the reducing end. A second acetyl group can be substituted on the C-3 or C-6 of the *N*-acetylglucosamine coupled to the non-reducing sugar. FAB$^-$-MS carried out before and after hydrogenation of the Nod metabolites showed components still differing by 2 mass units, indicating that this is not due to the presence of unsaturated fatty acids. Monomeric sugars were obtained after peracetylation of the Nod metabolites followed by hydrolysis of the β-(1,4) bridges. GC-MS indicated that the two supplementary mass units may be due to a reducing end sugar without ring formation or a glucosaminitol, which could be acetylated.

BR816 possesses one copy of *nodSU* and *nodH*, encoding proteins involved in methylation, carbamoylation and sulfation of the Nod factor backbone. In BR816, one *nodFE* copy has been characterised and hybridisation studies with heterologous *nodEF* genes suggest the possibility of two more *nodEF* copies (van Rhijn, unpublished). The *nodEF* genes likely determine the nature of the acyl chain linked to the core molecule (Spaink et al., 1991), however, no polyunsaturated fatty acids were detected. A gene encoding an acetyltransferase has not yet been identified. We will now this system to test the functionality of known nodulation genes in BR816. The discovery of a new type of Nod factor molecule opens up the search for new genes in the *Rhizobium* signalling.

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IDENTIFICATION OF PUTATIVE SIGNAL PEPTIDASE SUBSTRATES IN *BRADYRHIZOBIUM JAPONICUM*

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Two strains 132 and 184 have been isolated from *Bradyrhizobium japonicum* 110*spc*4 after Tn*phoA* insertion. The gene disruptions occurred in two distinct genes encoding signal peptidases. Their functions were demonstrated by complementing the strain IT41 of *E. coli* (Müller et al., 1995a ; Bairl, Müller, 1998). In symbiotic association with *Glycine max*, these mutant strains of *B. japonicum* present altered symbiotic phenotype : acetylene reduction is decreased and bacteroids are collapsed in differentiated nodules (Müller et al., 1995b). Moreover in soybean nodules infected by the strain 184, the pattern of symbiosome proteins is altered : the concentration of a 53 kDa protein is reduced in mutant nodules. The corresponding cDNA sequence was cloned and presents no significant similarity to other sequences. But the gene expression shows that it also reduced in mutant nodule and reveals a nodulin gene (Winzer et al., 1999). These drastically different phenotypes are probably caused by deficiencies in protein maturation of the microsymbiont during nodule organogenesis. Based on this assumption, several experiments were started in order to identify and isolate such signal peptidase substrates (or preproteins). Methodologies have been developed from bacterial liquid culture for isolating proteins contained in cytosol, membranes, periplasm and cell-free medium. These different cell fractions have been analysed by gel electrophoresis.

By using 2-D SDS-PAGE, *cytosolic fractions* of the different strains presented different accumulated proteins. Some of them could be substrates for the signal peptidases SipS and SipF when such proteins are present in the mutant strains and absent in the wild type strain. *Proteins extracted from the periplasm* presented promising pattern on 1-D gel. One band of 74 kDa is not present for the 2 mutants strains, another band of 37 kDa is also absent in the strain 132. Fermenter cultures have been developed in order to obtain sufficient amount of protein extracts for 2-D gel analysis. *Membranes protein* extracts separated on 1-D gel did not reveal significant differences. Moreover for testing if cytochromes *b* and c_1 are substrates of signal peptidase (Thöny-Meyer et al., 1991), duplicate gels were also heme stained. Similar protein patterns were obtained with the different strains. The maturation of the cytochromes *b* and c_1 is not affected in the strains 132 and 184. No differences between the three strains could be detected by 1-D gel analysis of the *secreted proteins* concentrated from the liquid culture medium.

Microsequencing will be the next step to obtain information about putative function of these differentially accumulated proteins in cytosol and periplasm. This also will give the opportunity for cloning the corresponding genes by reversed genetics.

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REQUIREMENTS OF *SINORHIZOBIUM MELILOTI* SURFACE POLYSACCHARIDES FOR AN EFFECTIVE NODULATION DEPEND ON THE SPECIES OF THE *MEDICAGO* HOST

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1. Introduction

A number of bacterial components are involved in legume nodulation and N2 fixation by rhizobia. Among them are extracellular (EPSs) and cell surface polysaccharides (PSs). Requirement for a PS may depend on the particular host-rhizobia interaction. Thus, while EPSs are necessary for invasion of indeterminate nodules, the lipopolysaccharides (LPSs) are required for the infection of determinate nodules (Diebold, Noel, 1988). Recently, the symbiotic role of Kdo-rich capsular polysaccharide (KPS) was described (Reuhs *et al*, 1995). Presently, the extent and host range of the functional complementation among different surface polysaccharides remains to be established. In this work, the symbiotic effect of single mutations in EPSI, LPS, or capsular polysaccharide on the nodulation of different *Medicago* species was investigated.

2. Materials and Methods

Polysaccharide mutants were constructed by plasmid integration mutagenesis. PCR products corresponding to internal portions of PS biosynthesis genes were ligated into the mobilizable suicide vector pk18mob. The resulting hybrid plasmids were transferred from the mobilizing strain *E. coli* S17-1 to *Sm*41. Cells that have integrated the recombinant plasmids into the genome were selected by the vector-encoded antibiotic resistence marker. Specific mutant phenotypes were analyzed in the selected transconjugates. As expected, the mutation of *exoY* resulted in loss of calcofluor-binding. Mutation of *lpsB* lead to an altered LPS mobility in SDS-PAGE and higher sensitivity of the mutant to detergents. Mutation of *rkpZ* resulted in slower mobility of KPS components in SDS-PAGE. Symbiotic analysis of PS mutants on *M. sativa* and *M. truncatula* involved thirty plants inoculated with each mutant. They were analyzed for number and position of root nodules, and for N_2-fixation.

3. Results and Discussion

None of the Sm41 mutants showed deficiencies in N_2-fixation on alfalfa. The EPSI mutant induced small, white, inefficient nodules on *M. truncatula* suggesting that EPSI is required for infection. Thus, EPSI cannot be replaced by any other polysaccharides naturally produced by strain Sm41, including EPSII and KPS (*rkpZ*$^+$). In agreement with this observation, EPSII could not functionally complement the defective nodulation of EPSI mutants on *M. truncatula*, using Sme 2011 derivative mutants. The supressor effect of KPS on EPS mutations appears functional on certain *Medicago* spp. Nodulation of *M. truncatula* by LPS mutants was dependent on bacterial genotype. Whereas *Sme* 2011 produced Fix$^-$, the *Sm41* did not. Double mutants will be used to investigate if the differential phenotypes of LPS mutants aong strains arise from the presence of the natural EPSII or KPS-*rkpZ*$^+$.

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ndvD, A NEW GENE INVOLVED IN CYCLIC ß-GLUCAN SYNTHESIS IN *BRADYRHIZOBIUM JAPONICUM*

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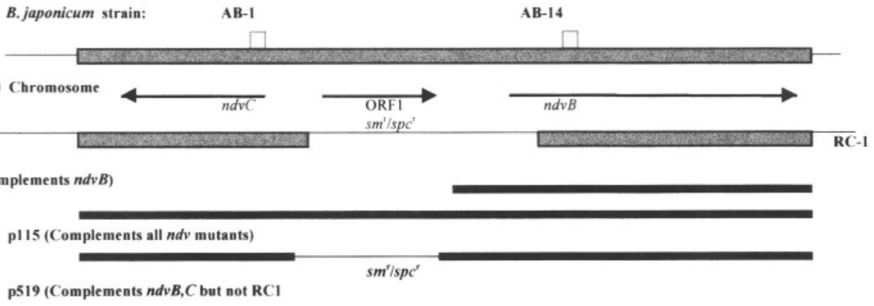
The soybean microsymbiont, *Bradyrhizobium japonicum* synthesizes periplasmic cyclic ß-(1,3),(1,6)-glucans during nodule development. The function of cyclic glucans in free-living bacteria is periplasmic osmoprotection (Breedveld et al., 1994). The role in symbiotic interactions remains unclear, but may involve suppression of host-defense responses (Bhagwat et al., 1999). We have characterized the cyclic glucan synthesis locus from *B. japonicum* and identified two genes (*ndvB,C)* required for glucan synthesis and symbiotic N-fixation (Bhagwat et al., 1995, 1996). Mutation of *ndvB* abolished glucan synthesis, while mutation of *ndvC* resulted in glucans with only ß-(1,3)-linkages. In addition to the symbiotic defect, the *ndvB* mutant is non-motile and hypoosmotically sensitive, whereas the *ndvC* mutant is motile and osmotically competent. *ndvB* appears to encode ß-(1,3)-glucosyl transferase and *ndvC* appears to encode ß-(1,6)-glucosyl transferase. Sequencing of the *ndvB,C* region identified an ORF between *ndvB* and *ndvC*. To determine the role of this ORF, we constructed a plasmid in which a 2 kb segment containing ORF1 and the promotor segment of *ndvB* was replaced by a 2 kb sm^r-spc^r cassette. This plasmid was used to create mutant strain RC-1 (Figure). RC-1 is non-motile, does not synthesize glucans and is symbiotically ineffective.


B. japonicum strain:
AB-1
AB-14
USDA110 Chromosome
ndvC
ORF1
ndvB
sm^r/spc^r
RC-1
p530 (Complements *ndvB*)
p115 (Complements all *ndv* mutants)
sm^r/spc^r
p519 (Complements *ndvB,C* but not RC1

By complementing RC-1 with various plasmid constructs containing combinations of *ndvB*, ORF1 and *ndvC*, we demonstrated that ORF1 is required for significant glucan synthesis and symbiotic effectiveness. So, we named it *ndvD*. Its deduced amino sequence has 62% identity to a serine threonine kinase in a segment of the gene, suggesting a regulatory function for *ndvD* which is upstream of *ndvB*.

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BORON-CALCIUM RELATIONSHIP IN NODULATION AND NODULE ORGANOGENESIS

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A relationship between Boron (B) and Calcium (Ca) in plants has been reported (Kowayashi et al, 1999). The level of both nutrients appeared inversely co-related so that increasing one of them determines a lower requirement of the other (Esteban et al, 1997). Because B has been demonstrated to be essential for the development of legume root nodules and symbiotic N2 fixation (Bolaños et al, 1994), we have focused our study on the investigation of the *Rhizobium*-legume association when plants of *Pisum sativum* (pea), *Phaseolus vulgaris* (bean), and *Medicago sativa* (alfafa) grow with several levels of B (from 0, -B to 5 ppm, 5B) and Ca (from 0, -Ca to 1,36mM, 2Ca).

Nitrogenase activity (measured as ARA) was influenced by the level of B and Ca. The inhibition caused by B deficiency was recovered by a supply of Ca, and the effects of low Ca or high toxic B (from 0.5-1 ppm) were also restored by the increase of B or the decrease of Ca respectively.

A clear effect was the increase of the number of nodules in plants treated with an extra supplement of Ca, being in most of cases double of tha obtained in Ca normal or in Ca deficient treatments. The capacity of root exudates to induce *nod* genes activity (measured as **β-gal** activity of *nodDp*::*lacZ* constructions) was higher in 2Ca treatments and can explain the effect on the amount of nodules.

Longitudinal sections of nodules, whose structure is extremely altered in the absence of B, showed a partial restoration by adding Ca (-B+2Ca) of the disorganized structure of –B nodules, so that irregular cell walls and tissue differentiation is recovered. Another interesting observation can be made in determinate bean nodules. B deficiency leads to an extremely high mitotic activity previously reported (Bonilla et al 1997) in the infected zone of –B+Ca treatments, possibly due to an increase of auxins mediated by B deficiency (Hirsch, Torrey 1980). Addition of extra Ca turns back this effect, which has to be investigated in more detail.

Following the evolution of nitrogenase activity in pea nodules can be observed that the absence of B provokes not only an inhibition but also a delay in reaching the maximum, suggesting that the development of the symbiosis is damaged and retarded at the same time. Addition of extra Ca (2Ca) inhibits nitrogenase of +B treatments but recovers the activity of –B plants, although the delay persists. However, measuring the activity per nodule there was not a significant increase in -B+2Ca nodules. Therefore, although the supply of Ca restored the structure of nodules, the recovered activity of plants should be mostly due to the increase of nodules more than to an effect on their development.

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EXPRESSION OF THE *BRADYRHIZOBIUM JAPONICUM NOLA* GENE IN RESPONSE TO CHEMICALLY DISTINCT SOYBEAN INDUCERS

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1. Introduction

Two inducers of the *Bradyrhizobium japonicum nolA gene* have been characterized by using noLA-lacZ fusions. These inducers were isolated from extracts of 5-day old etiolated soybean seedlings. The first is peptide-like and may act only on the *nolA* P1 promoter. The second is chitinase sensitive and induces expression of *nolA* from the P2 promoter. *B. japonicum* strains treated with both compounds nodulated soybean plants to a higher level than with untreated *B. japonicum* strains.

2. Results and Conclusions

I: NolA is induced by two chemically distinct compounds. Soybean seedling extracts (SSGE) were fractionated by reverse phase HPLC over a C-18 column (Phenomenex). Bound samples were then eluted with a methanol solvent gradient and analyzed for *nolA* inducing activity (Loh,J et al 1999). Fractions collected at 40 and 55 minutes, indicated as INDUCER-1 (IND-1) and INDUCER-2 (IND-2), were able to induce expression of *B.japonicum nolA-lacZ* fusions.

II: IND-1 and IND-2 are sensitive to proteinase K and chitinase treatment, respectively. Purified active fractions (IND-1 and IND-2) were assayed for proteinase and chitinase sensitivity. Results showed a strong sensitivity of IND-1 to proteinase K, indicating its possible protein-like nature. Conversely, the sensitivity of IND-2 to chitinase suggests a chitin-like structure for IND-2.

III: IND-1 and IND-2 act separately on the P1 and P2 promoters. To analyze the role of IND-1 and IND-2 on *nolA* expression, both compounds were compared by its ability to induce the NolA_1 and NolA_3 lacZ-fusions. As previously mentioned, expression of NolA_1 occurs from the P1 promoter, while NolA_3 is expressed from the P2 promoter (Loh et al, 1999). The results showed a higher activity of nolA 1,2,3 strains with both Ind-1 and Ind-2.

IV: Effect on nodulation of soybean cultivars. Fractions of SSGE from soybean cultivar UFV-16 were applied to a silica gel column and eluted sequentially with ethanol, methanol, ethyl acetate and chloroform. The eluted fractions were evaluated for **β-gal** activity using a ***nolA**$_{1,2,3}$**-lacZ*** fusion (Garcia et al, 1996). Active group fractions termed G2 (chitin-like) and G10 (peptide-like) were tested for their effect on nodulation with growth pouches using *B. japonicum* strains that were either untreated or pretreated with G2 or G10. The results indicate that pretreatment of *B. japonicum* cells with both G2 and G10 increase the capacity of *B. japonicum* to nodulate soybean plants.

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IS ETHYLENE INVOLVED IN NODULE INITIATION ON *SESBANIA ROSTRATA?*

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During the symbiotic interaction between *Sesbania rostrata* and *Azorhizobium caulinodans* ORS571, infection pockets are formed in the outer cortex and later on also in the inner cortex. These infection pockets are large intercellular spaces occupied by proliferating bacteria. An electron microscopic analysis showed that infection pocket formation is accompanied by local plant cell death. Cells in infection pocket regions were often shrunk or completely flattened and contained fragmented vacuoles and cytoplasmic electron-dense material, which is presumably a sign of protein aggregations. The plasma membrane became detached from the cell wall and cells were separated from neighboring cells at the level of the middle lamella. Furthermore, it has been demonstrated that lipochitooligosaccharide Nod factor signal molecules are required for infection pocket formation.

Previously, purified Nod factors have been reported to induce the formation of dense root hair bushes at lateral root bases of *S. rostrata,* corresponding to the nodulation sites. These root hair bushes consist of deformed root hairs and are also formed 15 to 24 hours after inoculation of roots with *A. caulinodans* ORS571, but not after inoculation with a *nodA* mutant that is unable to synthesize Nod factors. In different plants, root hair formation has been shown to be mediated by the plant hormone ethylene. Indeed, silver ions, which are widely used as inhibitors of ethylene perception, or (2-aminoethoxyvinyl)-glycine, which is an inhibitor of the 1-aminocyclopropyl-l-carboxylic acid synthase involved in ethylene synthesis, blocked Nod factor-induced root hair formation, whereas exogenous ethylene induced the formation of root hair bushes. In the latter case, the efficiency of root hair bushes formation at lateral root bases was rather low and root hair growth was also observed on other parts of roots, which was not seen when treated with Nod factors. These observations suggest that Nod factors induce a local ethylene production. Interestingly, diphenyleneiodonium, an inhibitor of NADPH oxidases that are involved in the production of reactive oxygen species, and ascorbic acid, a scavenger of hydrogen peroxide, which may be released during an oxidative burst, inhibited Nod factor-induced root hair formation as well. Furthermore, nodule initiation was completely blocked either by inhibitors of ethylene perception and synthesis or by inhibitors of oxidative burst induction. Concentrations of inhibitors were chosen in such a way that no severe effects could be observed on plant and bacterial growth, or on Nod factor production or secretion *in vitro.*

These observations lead us to propose that Nod factors may stimulate a local reactive oxygen species-mediated ethylene production that may be required for nodule initiation. Indeed, either reactive oxygen species or ethylene or both may be involved as part of a signal transduction cascade triggering a local plant cell death program and consequently create intercellular space (and a release of nutrients?) to form infection pockets, probably a *conditio sine qua non* for further invasion of the plant tissue.

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MUTATION IN PEA (*PISUM SATIVUM* L.) SYMBIOTIC GENE *Sym31* AFFECTS THE EXPRESSION OF *RHIZOBIUM LEGUMNOSARUM* BV. *VICIAE* VF39 GENE *dctA* AND NOT THAT OF *fixNc*

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The following bacterial transcriptional gene fusions were used: (i) constitutive-*lacZ* (*Km-lacZ* fusion in plasmid pGD499; Gitta et al., 1985), (ii) *dctA-lacZ,* (*dctA-lacZ* fusion in plasmid pCR12; Ronson et al., 1987), (iii) constitutive-*gusA* (**fixK**::**Gm**^{R}; U. Priefer et al., unpublished), (iv) *fixNc-gusA* (Schluter et al., 1997). The folowing pea mutant lines were used: (i) E135f (*sym13*) (Kneen et al., 1990), (ii) **Sprint-2Fix**$^-$ (*sym31*) (Borisov et al., 1992) and (iii) RBT (*sym13, sym31*) (Borisov et al. 1997) blocked at late stages of nodule development. Control lines were Sparkle and Sprint-2.

The pattern of *dctA-gene* expression is very much similar to that of constitutive ones in nodules of all mutant lines. Low-oxygen induced expression (beginning from interzone II-III toward deeper cell layers) of *fixNc-gene* was confirmed. The nodules of mutants **Sprint-2Fix**$^-$ (*sym31*) and RBT (*sym13, sym31*) have no distinct interzone II-III. It can reflect changes in oxygen barrier earlier revealed for the mutant in the gene *sym31* (Romanov et al., 1995) The changed oxygen barrier and histological nodule structure can cause differences in the expression pattern of *fixNc-gusA* transcriptional fusion in nodule of these lines. The nodules of mutant E135f (*sym13*) are characterized by premature degradation of nodule tissues. Early appearance of zone IV can change the pattern of all fusions used. Both constitutive fusions and *dctA-lacZ* were increased in their activity in "bacteroids" from mutant lines comparing to WT ones. In lines carrying mutation in the gene *sym31* this was much more pronounced. The number of recoverable bacterial cells from nodules in various lines had very similar trends to that of constitutive and *dctA-lacZ* fusions. The latter fact can be the reason of higher rate of the activities - undifferentiated bacteroids have higher level of *dctA*-gene expression. Quantitative analysis revealed no differences in activity of *fixNc-gusA* fusions in "bacteroids" isolated from WT and mutant lines.

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Acknowledgements

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ILVC MUTANTS REVEAL NOVEL COMPLEXITY ON SIGNAL EXCHANGES BETWEEN *SINORHIZOBIUM MELILOTI* AND ALFALFA

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We have reported that a mutation in the ilvC gene of *S. meliloti* 1021 determines a symbiotically defective phenotype (Aguilar, Grasso, 1991). This gene encodes the enzyme, acetohydroxyacid isomeroreductase, which catalyses the second step in the biosynthetic pathway for isoleucine and valine. Although this mutant is able to induce root hair deformation on alfalfa roots (Hirsch et al, 1982), we find that nodulation genes *nod*ABC are not activated *in vitro* by the inducer luteoline. Here we examine the symbiotic behaviour of IlvC$^-$ derivatives of three strains of *S.meliloti* (SmL5-30, Sm41 and Sml021). All three mutants were symbiotically defective, however differences in nod promotor activity were found among them. The IlvC$^-$ derivatives of Sml021 and Sm41 showed a significant decrease in the activity of the *nod*ABC promoter (less than 10%), whereas in the mutant IlvC$^-$ of SmL5-30 it is reduced to 50%.

The production of Nod factors by the IlvC$^-$ strains was investigated by *in vivo* radioactive labeling and thin layer chromatography (TLC) analysis. The results indicate that *ilvC* mutation reduces Nod factor production without affecting their sulphation. The early events induced by the IlvC$^-$ strains on alfalfa roots were examined. Alfalfa roots inoculated with the IlvC- strains showed abundant hypertrophied root hairs, only exceptionally curled root hairs, but no infection threads. Four weeks after inoculation, no nodules were detected whereas thick roots, surrounded by long and deformed root hairs, were frequently observed. No differences in these plant responses were found among the mutants. Therefore, IlvC- strains induce certain early responses on alfalfa roots but are unable to form infection threads irrespective of their nod-promoter activity. The plant response to inoculation with IlvC$^-$ strains was investigated by monitoring cortical cell activation (CCA) (Ardourel et al, 1994). Seven days after inocculation with *ilvC* mutants, cortical cells accumulating starch were detected in secondary roots. In this zone, cellular clusters with intense amyloplast accumulation consisting of small cortical cells separated by periclinal septa was observed. This pattern of starch accumulation was different to that induced by wild type or Exo$^-$ mutants strains. However, by using a double *nodC/ilvC* mutant, we showed that starch accumulation is a result of *nod* gene expression.

To assess whether IlvC- strains can be extracellularly complemented during infection, we performed coinoculations with an *exoY* and a *nodC* strain. Coinoculation of the *ilvC* mutants and *nodC* mutant resulted in the same plant response as with *ilvC* mutants alone. In addition, roots inoculated with *ilvC* plus *exoY* mutants showed empty nodules identical to those induced by the latter strain. However, coinoculation with *exo*Y and *nodC* mutants formed nodules similar to those with wild type. Further coinoculation experiments using the double mutant strain *nodC/ilvC* in a mixture with the *exoY* mutant gave nodules similar to those found with the exoY mutant. This result indicates that the *ilvC* mutation negatively affects the ability of Nod$^-$ strain to complement the Exo$^-$ strain. Therefore we propose that the *ilvC* gene plays alternative roles to Nod signalling in symbiosis.

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CHARACTERIZATION OF A FUNCTIONAL NIFA-σ^{54}-TYPE PROMOTER UPSTREAM OF THE *AZORHIZOBIUM CAULINODANS NODA* GENE

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To study the invasion of *Azorhizobium caulinodans* ORS571 during nodule development on *Sesbania rostrata,* pRG960SD-32 carrying a ß-glucuronidase (GUS)-encoding gene under the control of the *Azorhizobium nodA* promoter region, was introduced into the wild-type strain. A common *nodA* promoter consists of a *nod* box to which the NodD transcription factor is binding in the presence of correct flavonoids, such as naringenin. We observed a persistent *nodA* promoter activity until approximately 20 days after inoculation. Because of this long lasting *nodA* promoter activity *in vivo,* a more extensive sequence analysis was performed revealing the presence of a NifA and a σ^{54} box upstream of the *nod* box. NifA-σ^{54}-type promoters control the expression of *nif* and *fix* genes that are expressed in bacteroids. Activation of NifA-σ^{54}-type promoters is mediated by a complex regulatory cascade and is tightly controlled by three different two-component systems, sensing several environmental conditions. To explain the above observations, it was tempting to investigate whether the putative NifA-σ^{54}-type promoter, upstream of the *nod* box promoter, was functional.

ORS571(pRG960SD-32) showed a relatively high GUS activity *in vitro* in micro-aerobic conditions in the absence of naringenin. This activity disappeared when the NifA-σ^{54}-type promoter was knocked out or when strains were grown at normal oxygen concentrations. Furthermore, this activity was naringenin and NodD independent, but NifA and FixK dependent. These data suggested the presence of a functional NifA-σ^{54}-type promoter controlling the expression of the *nodA* operon.

Previously, it has been shown that coinoculation of a *nodA* mutant (Nod$^-$) with ORS571-X15, a surface polysaccharide mutant (pseudonodules), led to the formation of functional nodules, the central tissue of which was occupied by the *nodA* mutant (D'Haeze et al. 1998). Introduction of pRG960SD-32 into ORS571-3.1D, a *nodD* mutant, resulted in an *Azorhizobium* derivative that upon coinoculation with ORS571-X15 induced functional nodules with blue central tissue after GUS staining. However, when the NifA-σ^{54}-type promoter was knocked out, a decreased GUS activity was observed. Quantitative GUS assays using isolated bacteroids confirmed these results and demonstrated that the NifA-σ^{54}-type promoter was indeed functional *in vivo.*

To determine whether the activation of the NifA-σ^{54}-type promoter during later stages of nodulation would play a role, a genomic mutant was constructed by deleting the NifA box and introducing a cassette in a point-mutated σ^{54} box. The mutations did not cause any effect on stem nodulation ability, but when plants were grown in high light conditions, root nodules became senescent significantly earlier. These results illustrated that the expression of the major *nod* operon of *A. caulinodans* is controlled by both a classical *nod* box promoter and by a functional NifA-σ^{54}-type promoter, suggesting a role for certain azorhizobial Nod factors during later stages of nodulation.

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POSSIBLE ROLE FOR DIAMINE OXIDASE IN INFECTION THREADS.

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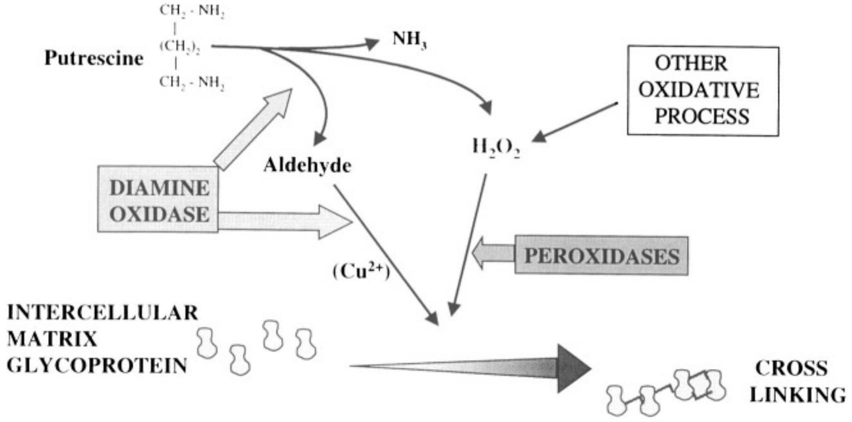
In pea, bacteria growing within infection threads are embedded in a plant-derived matrix containing an extensin-like glycoprotein recognised by monoclonal antibody MAC265. Because this same matrix glycoprotein (MGP) is exuded by the cells at the root tip, we have been able to show that it apparently becomes cross-linked in the presence of hydrogen peroxide (5mM). Known inhibitors of peroxidase (e.g. salicyl hydroxamate) increase the extractability of MGP from root tips. We postulate that the control of MGP cross-linking could be an important factor regulating whether or not infection threads abort. Similarly, cross-linking of cell wall extensins has been widely reported as a rapid response to plant pathogens.



We have examined the possible involvement of pea diamine oxidase (DAO) as a potential source of hydrogen peroxide (H_2O_2). This enzyme, which is involved in polyamine catabolism, oxidises putrescine and cadaverine with the formation of the corresponding aldehyde, H_2O_2 and ammonia. Using immunolocalisation techniques, diamine oxidases have been found in the apoplast of mature tissues, for example in sections of pea epicotyl. It has been proposed that DAO may have a special role in the localised production of H_2O_2 from polyamine substrates. In turn, this H_2O_2 can be utilised by peroxidase enzymes present in the apoplast, thus leading to the modification of cell wall plasticity.

When putrescine, the preferred substrate for DAO, was applied to pea roots for 5 min, it caused a total disappearance of MGP antigen from the western blot of root exudate. This effect was reversed by *o*-phenanthroline, a specific inhibitor of DAO. By contrast, histamine and spermine, which are less effective as substrates for pea DAO, induced only a partial disappearance of MGP. Thus the polyamine-induced insolubilisation of MGP at the root surface correlates closely with the preferred substrate activity for DAO. Application of putrescine (1mM) is inhibitory to nodule development. Further analysis of the nodulation process will involve transgenic lines of pea and *Medicago truncatula* carrying the promoter of PsENOD12A fused to the sense or antisense coding sequence for DAO.

CYSTEINE PROTEASE LOCALISATION IN PEA ROOT NODULES

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Cysteine protease encoded by *PsCyp15a* has been identified as a transcript up-regulated in wilted pea shoots (Jones and Mullet 1995) and more recently in pea nodules (Kardailsky and Brewin 1996). Using specific molecular probes and transgenic plants, we have investigated the cellular and subcellular localisation of this protease.

The coding sequence (lacking leader and pro peptides) was expressed in *E. coli* using the pRSET expression system (Invitrogen). Polyclonal antiserum raised against recombinant PsCYPl5A protein was used to identify the antigen in western blots of pea tissue extracts. In nodules and other tissues two bands were observed (at ca. 40kDa and ca. 31kDa), apparently corresponding to pro- and mature forms of protease. Using confocal and electron microscopy, PsCYP15A antigen was localised in vacuoles and small cytoplasmic vesicles. In infected nodule cells, it was also present in the perisymbiont space. However, antigen was not found in infection threads or intercellular spaces.

PCR-based cloning of the promoter region of *PsCyp15a* was accomplished using restriction-digested adaptor-ligated genomic DNA, together with gene-specific primers. In order to investigate gene expression during nodule development, promoter:gus fusions were generated in the transformation vector pE6NOS*Bar,* using short (260bp) and long (900bp) segments of the promoter. Transgenic hairy roots were established on *Vicia hirsuta* following inoculation with *Agrobacterium rhizogenes* and these were induced to nodulate with *Rhizobium leguminosarum* bv. *viciae.* Results showed that a promoter region of ca. 900bp was sufficient for spatial expression similar to that observed previously by *in situ* hybridisation studies. With this construct, GUS expression was observed in nodule primordia (5dpi). In nodule sections there was strong expression in apical tissues (both infected and non-infected), and a slightly lower level of expression in host cells containing mature bacteroids. These findings support a developmental or adaptive role for this protease in host cells during nodule development. This may correlate with osmotic adaptation of pea cells in wilted shoots.

A homologue corresponding to *PsCyp15a* was isolated from a cDNA library derived from nodules of alfalfa (*Medicago sativa*). The two sequences showed 92% homology in deduced amino acid sequence and the two genes showed synteny in their RFLP map positions (N. Ellis and G.B. Kiss pers. comm). Experiments are in progress to investigate the functionality of *MsCyp15a* in transgenic lines of *M. truncatula.*

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OXALATE OXIDASE ACTIVITY IN PEA ROOT NODULES

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Oxalate oxidase (OxOx) is a cell wall enzyme which degrades oxalate and produces H_2O_2. We are investigating the possible implication of OxOx as a local source of peroxide for cell wall hardening during tissue invasion by *Rhizobium.* OxOx is a glycoprotein belonging to the germin family in cereals, such as barley (Woo EJ et al 1998). Although germin-like proteins have been reported in dicotyledonous plants, none of these proteins has been reported to have OxOx activity. However, we now report biochemical evidence for an SDS-resistant heat sensitive OxOx activity in pea root and nodule extracts.

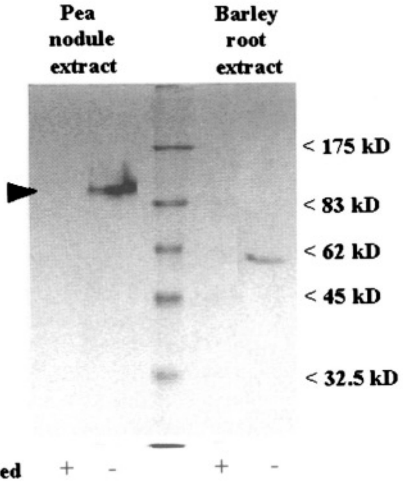

Figure. 1. *Demonstration of heat-sensitive OxOx activity in pea nodule extract after SDS-PA gel electrophoresis. Reaction mixture was: 2mM Oxalate, 5U/ml horseradish peroxidase in 40mM succinate buffer (pH 3.8) with 60% ethanol. (Note that the molecular weight standards are for pre-heated protein samples.)*

We have raised a rat antiserum against the wheat OxOx monomer (expressed in *E. coli)* and demonstrated that it recognises a 30kDa monomer and (apparently) an inactive multimer from pea extracts. However, the only form of the cereal protein to have enzyme activity is the hexamer. This form was not well recognised by the antiserum on western blots from barley extracts and nothing corresponding to this was observed in samples from pea extracts. By coincidence, a 30 kDa protein belonging to the germin family was recently isolated from pea seedling extracts because it promoted attachment of *Rhizobium* (and *Agrobacterium*) to pea root hairs (Swart S et al 1994). The plant protein was shown to overcome the inhibitory effect of an excess of rhicadhesin, a bacterial attachment protein. This bioassay was used as the basis for purification of the protein. The N-terminal sequence shows homology with cereal germins. Using degenerate primers derived from this sequence, we have obtained the cDNA from pea nodule RNA. The deduced peptide sequence shows >50% homology with wheat OxOx, and includes most of the conserved residues that are thought to be essential for enzyme activity. RT-PCR showed that this gene is expressed in roots, nodules, shoots and leaves.

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PEA (*PISUM SATIVUM* L.) GENE *SYM33* CAN PLAY A ROLE IN ETHYLENE DEPENDENT REGULATION OF NODULATION

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1. Introduction.

The pea mutant **SGEFix$^-$-2** in gene *sym33* obtained after EMS mutagenesis (Tsyganov et al., 1994) is blocked at the stage of infection thread differentiation in symbiotic nodule tissue - no infection droplet formation and endocytosis of bacteria (Tsyganov et al., 1998). In addition, decreased nodulation is also a characteristic. This mutant phenotype is a leaky one. Sometimes in some nodule cells the endocytosed bacteria can be found. Moreover, sometimes pinkish nodules can be formed on the roots of this mutant and symbiosomes with abnormally differentiated bacteroids are observed in the infected cells (Tsyganov et al., 1998).

2. Results and Discussion

2.1. Temperature was suggested and checked to be an agent affecting this phenotype. It was shown that the most numerous nodules are formed at 15°C and increase of temperature determines decrease in nodulation, at 27°C nodules are not formed at all. Number of pinkish nodules described above was highest at optimal temperature, 21°C (about 20% of total amount). Initial line SGE decreases number of nodules twice at 27°C and has highest number of them at optimal temperature.
2.2. Decreased nodulation and temperature dependence suggested that ethylene based regulation of the root/nodule can be involved in determination of this mutant phenotype (Fearn, LaRue, 1991). Four different agents (Ions **Ag$^+$**, AVG, ACC, Ethephon) that change the ethylene biosynthesis/action were used to check this hypothesis. It was shown that ethephon has the highest effect on nodulation in both line SGE and mutant **SGEFix$^-$-2** (*sym33*) by increasing the weight of one nodule. It suggests that ethylene can regulate not only nodule meristem initiation (Heidstra et al., 1997) but also prolonged action of nodule meristem. Also it was shown that mutant **SGEFix$^-$-2** (*sym33*) has stronger although unexpected effect (decreased number of nodules and total and single nodule weight) on AVG treatment than initial line SGE. The latter fact means that pea symbiotic gene *synr33* can play a role in ethylene dependent regulation of nodulation.
2.3. Gene *sym33* was localised in linkage group I. Previously in this linkage group a number of pea symbiotic genes including *sym5* and *ENOD40* which take part in hormone based regulation of nodulation were localised (Weeden et al., 1998).

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ANALYSIS OF *MEDICAGO TRUNCATULA AUX1*-LIKE GENES, ENCODING PUTATIVE AUXIN INFLUX CARRIERS: ARE THEY INVOLVED IN *RHIZOBIUM*-INDUCED NODULE ORGANOGENESIS?

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1. Introduction

Development of the nodule organ involves changes in hormones, including auxin (Hirsch, Fang 1994). Recently it has been shown using an auxin responsive reporter construct that inhibition of polar auxin transport is an early event in nodulation, mediated by addition of Nod factors. This inhibition of auxin tranport is then followed by an accumulation of auxin at the site of nodule initiation (Mathesius et al. 1998). In root development it has been shown in *Arabidopsis* that the auxin influx carrier AUX1 (Marchant et al. 1999) is involved in the determination of the number of lateral roots and is expressed at the sites of lateral root initiation (A. Marchant, MJ Bennett, personal communication). We have cloned *AUX1* homologues from the model legume *M. truncatula* in order to study whether they may also be involved in nodule initiation.

2. Results

Clones corresponding to five *AUX1*-like genes have been isolated from root and nodule cDNA libraries. The encoded proteins show over 85% amino acid identity to the *Arabidopsis* AUXl protein and therefore these proteins probably perform similar functions. Northern blots indicated that the *M. truncatula AUX1*-like genes are expressed at low abundance in roots and nodules and a quantitative RT-PCR assay did not reveal any specific changes in the expression of the three most-highly expressed genes during early stages of nodule development. For this reason, in situ hybridisation was used to study whether spatial changes in their expression occur. This technique revealed that *AUX1*-like genes are expressed in root tips and developing secondary roots and during the early stages of nodule development. In particular, expression was detected in the pericycle and inner cortex of the very young developing nodule.

3. Conclusion

Our results support a model in which nodule initiation involves a transient inhibition of polar auxin transport (Mathesius et al. 1998) followed by a redirection of auxin flow towards the developing nodule, mediated by AUXl-like proteins.

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REGULATION OF *RHIZOBIUM TROPICI* CIAT899 *NODHPQ* GENES AND ITS ROLE IN HOST RANGE DETERMINATION

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Nod factors sulphation is required for biological activity and plays an important role in determination of the host range specificity. Therefore, nodulation on alfalfa by *Sinorhizobium meliloti* is abolished by mutation in sulphation genes, *nodH, nodP* and *nodQ.* However, *Rhizobium tropici* produces a sulphated Nod factor but it is unable to nodulate alfalfa. There might be specific receptors in roots which recognize each different Nod factors substitution. In alfalfa, it has been proposed the existence of a highly specific receptor recognizing the sulphated *S. meliloti* LCO which may be involved in infection processes, root hair curling and activation of cortical cells division. Molecular markers induced during first stages of nodule formation are known as early nodulin genes (*enod*). Many of them are induced by purified Nod factors. One of the first detectable early nodulin gene expression is *enod40.* We used it as a marker of nodulation induction after treatment with sulphated and non sulphated Nod factors produced by the wild type strain *R. tropici* CIAT899 and *nodHPQ* genes mutants of this strain. We have also studied whether Nod factor sulphation is under the influence of regulator *nod* genes, such as *nodD* or *syrM.* Influence of Nod factors modification on *R. tropici* host range determination was also studied. In this work we tried to determine the effect of *R. tropici* Nod factors in *Medicago sativa enod40* induction. Analysis of results indicated the presence of a low level of *enod40* expression in uninoculated roots of *M. sativa.* Roots treated with 10^{7} M *S. meliloti* Nod factors showed an important accumulation of *enod40* mRNA, which is normally accompanied by nodule primordia formation. However, no significant accumulation of *enod40* mRNA was seen after treatment with the same amount of *R. tropici* CIAT899, *nodP* mutant and *nodH* mutant Nod factors, suggesting that purified *R. tropici* Nod factors are either unable to induce alfalfa *enod40* gene expression or that basic levels of expression in root tissues do not allow the detection of gene induction. Expression analysis of *nodP::lacZ* transcriptional fusion in the wild type *R. tropici* CIAT899 and in the cured strain RSP900 suggested the existence of one or more regulator genes located in pSym that may control sulphation genes expression. In order to determine whether *R. tropici* CIAT899 Nod factors sulphation is controlled by this gene, we have designed primers from *Rhizobium* sp. NGR234 *syrM gene* DNA sequence, and we have tried to locate it in *R. tropici.* PCR analysis showed no amplification when *R. tropici* DNA is used as template. This result, added to the absence of hybridization when we used *Rhizobium* sp. NGR234 *syrM* gene as probe, suggest that probably *R. tropici* does not have this gene. These data lead us to assume that there may be another regulation system controlling Nod factors sulphation and therefore host range determination. In order to understand the role of Nod factors modification in host-range determination, we have transferred *S. fredii* fucosylation *nod* genes to *R. tropici* strains. TLC analysis of LCO produced by these transconjugants showed that only those carrying complete fucosylation operon were capable to change LCO profile. FAB analysis of the two new peaks that were collected from HPLC study, indicated the presence of methylfucosylated LCO in Nod factors produced by both trasconjugants. We also showed competition between sulphation and fucosylation *nod* genes but we have never found a sulphated methylfucose substitution, suggesting the specificity or high affinity of NodH to sulphate C6 of Nod Factors. Transconjugants were unable to nodulate soybean, habitual host of *S. fredii.* Thus, the determination of host-range is not only fucose-dependent.

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EXPRESSION OF A *MEDICAGO TRUNCATULA* APYRASE GENE IS INDUCED BY RHIZOBIA

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Lectins have long been implicated in the legume nodulation process but their precise role has remained controversial. Recently, Etzler et al. (1999) reported the isolation of a unique lectin (DB46) from the roots of the legume *Dolichos biflorus.* This lectin was found to bind to Nod signals from a variety of rhizobia but showed the highest apparent affinity for the lipo-chitin oligosaccharides (LCO) produced by *B. japonicum* and *Rhizobium* sp. NGR234. In addition to binding LCO, Etzler et al. (1999) found that DB46 had apyrase (i.e., NTPase) activity that was significantly stimulated upon addition of Nod signal. For this reason, the lectin was termed a lectin-nucleotide phosphohydrolase (LNP). The *D. biflorus* LNP (i.e., DB46) was found on the surface of root hairs and, indeed, addition of antibody to DB46 inhibited nodulation. The properties of this protein suggest that it could play a role in Nod signal recognition and transduce this binding signal by way of its apyrase enzyme activity.

A full-length cDNA clone encoding a putative *M. truncatula* apyrase was isolated by PCR amplification using primers to conserved motifs found in all apyrase genes. The sequence of the cDNA predicted an encoded protein of approximately 46 kDa (MT46a). Northern hybridizations indicated that the MT46a gene is expressed in roots. Using a gene specific probe made from the 3' end of the cDNA, northern hybridizations demonstrated that MT46 mRNA was induced very rapidly (i.e., within 3 hours) in roots inoculated with *Sinorhizobium meliloti.* Therefore, MT46a is an early nodulin. In addition to the cDNA for MT46a, a partial cDNA clone was also isolated and termed MT46b. Using northern hybridization to mRNA from both inoculated and uninoculated roots, we screened a large collection of *M. truncatula* mutants defective in nodule formation. Several of these mutants showed either a defect in the ability to induce MT46 mRNA upon inoculation or a significant reduction in the level of uninduced expression.

We have mapped the location of MT46a on the *M. truncatula* genetic map. A collection of 20 overlapping BAC clones of *M. truncatula* DNA form a contiguous region around the MT46a gene. End sequencing of the BAC clones revealed the presence of at least two additional apyrase genes adjacent to MT46a. Therefore, this cluster contains at least 3 distinct, apyrase genes. The MT46b apyrase gene has not been precisely mapped but is apparently unlinked to the apyrase cluster containing MT46a.

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SECTION V

STRUCTURE AND DYNAMICS OF DIAZOTROPHIC AND PLANT GENOMES

GENOME STRUCTURE OF DIAZOTROPHS

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The aim of genomics is the understanding of integral genome structures. It covers the obtention of the physical map and the nucleotide sequence of a genome or a comprehensive region of a genome, the analysis of sequences by informatic tools, and the experimental approaches to link the structure with the evolution, the function and the dynamics of genetic information.

Nitrogen fixation is present in some members of two of the three domains of biology: archae and bacteria. Among bacteria the capacity to fix nitrogen is found in very different organisms including anaerobes, aerobes, photosynthetic bacteria, blue-green algeae and actinomyces. Some bacteria fix nitrogen in the free-living state while others fix nitrogen in symbiosis with plants. Therefore, very different type of genomes may harbor nitrogen fixation and symbiotic genes. Moreover, in some cases such genes are present in the chromosome while in others are plasmid born. Lateral transfer of genetic information has been proposed to account for the scattered distribution of these genes among prokaryotes.

The state of the art of genome sequencing projects can be consulted in a web address (http://www.ncbi.nlm.nih.gov/Entrez/Genome/o.html). Up to September 1999, 21 prokaryotic genomes had been completely sequenced, annotated, and made available. Of these, the only microorganism that fixes nitrogen is the archeae *Methanobacterium thermoautotrophicum* (Smith et al, 1977).

Two whole genome projects in nitrogen fixing bacteria are now being pursued: *Rhodobacter capsulatus* and *Sinorhizobium meliloti*. *Rhodobacter capsulatus* is a purple non-sulphur photosynthetic bacterium that is able to fix nitrogen. The versatility of this organism, that can grow either photo or heterotrophically and either from atmospheric or combined nitrogen, makes it an ideal model to study these processes. A complete physical and genetic map of the model strain SB 1003 has been obtained (Fonstein, Haselkorn 1993) and the sequence of a continuous region of 189 kb has been published (Ulcek et al, 1997). The nucleotide sequence of the chromosome is now essentially completed (R. Haselkorn, personal communication).

An international effort aiming at establishing the complete nucleotide sequence of the genome of *Sinorhizobium meliloti* has started. The genome of this microorganism is constituted by three replicons of 3.6 Mb (chromosome) 1.7 Mb (p*SymB)* and 1.4 Mb (p*SymA*). Physical and genetic maps of the three replicons have been obtained (Honeycutt et al, 1993; Capela et al, 1999). The international genome sequencing project of *S. meliloti* is organized according to the structure of the genome. An european consortium led by Prof. F. Galibert is responsible for sequencing the chromosome; Prof. S.R.Long at Stanford University is responsible for the p*SymA* project; the sequencing of p*SymB* is organized as a joint project between Prof. A. Pühler at the University of Bielefeld (Germany) and Prof. T. Finan and Prof. B. Golding at MacMaster University (Hamilton, Canada). All three projects are expected to be completed by the end of year 2000.

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This will be of great relevance in the field of symbiotic nitrogen fixation since *S. meliloti* is the best known *Rhizobium* in regard to genetics and molecular biology. The advance of the project can be consulted in the following web addresses (http://cmgm.stanford.edu/~mbarnett /ixgenome.html) (http://sequence.toulouse.inra.fr/meliloti.html).

Important advances have been made in regard to the sequences of symbiotic regions of the *Rhizobium* genome. The complete nucleotide sequence of one symbiotic plasmid, that of the broad host range *Rhizobium* sp NGR234, has been obtained (Freiberg C et al, 1997). With this pioneering work, symbiotic nitrogen fixation entered the era of genomics. In addition to the direct data obtained in regard to the genes encoded by this plasmid, important general features were revealed. A large amount of the information present in the plasmid (18%) corresponds to IS like elements; the overall structure of the plasmid presents clusters of functionally related genes separated by IS elements. Homology searches and the analysis of the G+C content of different regions suggest recent lateral transfer of genetic information among related organisms.

The symbiotic plasmid of *Rhizobium etli*, the symbiont of bean, is on its way to completion (see Dávila et al, this volume). About 90% of the sequence of the 400 kb replicon has been obtained. Comparison of this sequence with that of the p*Sym* of *Rhizobium* sp NGR234 reveals that the number of orthologous genes shared by both plasmids is about 35% and that there is a large divergence in the relative positions of these genes.

In some cases, the symbiotic regions are present in the chromosome. In *Bradyrhizobium japonicum,* the symbiont of soybean, the nodulation and nitrogen fixation genes are clustered within a region of about 400 kb. The nucleotide sequence of this region is nearly completed (see Göttfert et al, this volume). In the case of a *Mesorhizobium* strain that nodulates *Lotus*, the symbiotic genes are present in a chromosomally integrated element or symbiotic island (Sullivan et al, 1995). This element transfers to non-symbiotic mesorhizobia in the environment and converts them to symbionts. The nucleotide sequence of this element is currently being determined (see Ronson et al, this volume).

With the advent of genomics, the center of molecular biology is moving from the gene to the genome. In this new biology the ideal model systems will be those in which the complete nucleotide sequence has been established. If we want to keep nitrogen fixation at the frontier of biologial research, a big effort should be made to obtain the DNA sequences of the genomes of several free-living and symbiotic nitrogen fixing organisms.

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ORGANISATIONAL, TRANSCRIPTIONAL AND FUNCTIONAL ANALYSES OF THE *RHIZOBIUM* SP. NGR234 GENOME

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1. Introduction

Rhizobium sp. NGR234 was the only fast-growing strain amongst thirty isolates extracted from nodules of *Lablab purpureus* growing in Papua New Guinea (soil pH 8.5) (see Trinick, 1980). Its host-range exceeds 112 genera of legumes and even extends to the non-legume *Parasponia andersonii* (Pueppke, Broughton, 1999). The genome of NGR234 is divided into three replicons – a 536 kb symbiotic plasmid, a ≈ 2 Mb mega-plasmid, and a ≈ 3.7 Mb chromosome (Flores et al., 1998). Plasmid transfer experiments indicated that most symbiotic genes are carried on the smaller of the two plasmids, pNGR234*a* (Broughton et al., 1984; Broughton et al., 1986). DNA sequence analysis confirmed these findings (Freiberg et al., 1997). Eighteen percent of pNGR234*a* is made up of insertion and mosaic sequences (IS/MS), suggesting that the genome is plastic. In addition to nodulation (*nod*), nitrogen fixation (*nif*), and fixation (*fix*) genes (involved in, e.g., bacteroid development) that are known to play roles in nodulation and nitrogen fixation, a locus encoding a type three-secretion system (TTSS) was also found (Viprey et al., 1998). Induction of the TTSS genes is flavonoid-dependent, but occurs later than nodulation genes (Perret et al., 1999).

2. Results and Discussion

Collectively, potential genes and gene-fragments comprise 78% of pNGR234*a* (416 out of 536 kb), but none of them are essential to transcription, translation or primary metabolism. On the other hand, all genes necessary for synthesis of rhizobial Nod-factors as well as elaboration of the nitrogen-fixing apparatus are present on this replicon (Freiberg et al., 1997). Putative transcriptional regulators comprise 24 of the predicted 416 open reading frames (ORFs). Computational analyses identified 19 *nod*-boxes and 16 conserved **NifA-σ^{54}** regulatory sequences, which are thought to co-ordinate the expression of *nod* and *nif* genes, respectively. In contrast to the *nod*-genes that are dispersed over the entire symbiotic plasmid, the *fix* genes form a 55 kb cluster (*fixU* to y4xE) which contains ten of the **NifA-σ^{54}-dependent** promoters. A group of genes that encode components of a bacterial TTSS is also present. In both animal and plant pathogens, the TTSS is an essential component of pathogenicity, and is responsible for injection of proteins into the eukaryotic cells. Secretion of at least five proteins (y4xL, NolJ and No1X as well as two others) by the TTSS occurs after induction with flavonoids. Polar mutations in two TTSS genes, *rhcN* and the *nod*-box controlled regulator of transcription y4xI, block the secretion of all five proteins, and strongly affect the ability of NGR234 to nodulate a variety of tropical legumes including *Pachyrhizus tuberosus* and *Tephrosia vogelii* (Viprey et al. 1998; H.B. Krishnan and C. Marie, unpublished). Both

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increases and decreases in the number of nodules and their capacity to fix nitrogen were observed, suggesting that in some plants the secreted proteins are perceived as elicitors of defence responses while in others they stimulate nodule development. We are currently investigating the possibility that the secreted proteins might be necessary for growth of the infection thread in some plants (see Perret et al., 2000).

Further delineation of symbiotic loci was obtained by determining which of the 416 ORF's (plus their inter-genic regions) produced flavonoid-inducible transcripts or mRNAs in nodules. Genes involved in the synthesis of Nod-factors (e.g., the three host-specificity of nodulation (*hsn*)-loci) are induced rapidly after flavonoid addition, while others thought to act within the plant (e.g., those encoding the TTSS machinery) respond more slowly. Important changes in gene expression accompany the differentiation of rhizobia into nitrogen-fixing bacteroids. Principally, these involve repression of the majority of flavonoid-inducible transcripts and induction of those required for effective nitrogen fixation. Transcriptional analyses suggest that many more loci are specifically expressed in bacteroids however. Some, like the cytochrome P450 operon (y4kR to y41D) are under the control of one of the six other **NifA-σ^{54}** like sequences. Two opposing operons, y4nG to y4nJ and y4nL to y4nM, which possibly encode functions linked to sugar metabolism, are also regulated in a similar manner. Many IS and transposon (Tn) like sequences are strongly expressed under all conditions. More diverse transcripts were found in bacteroids of determinate as opposed to indeterminate nodules.

Random sampling of the rest of the genome suggests that far fewer (2.2%) IS/MS elements are present on the larger plasmid and the chromosome than on pNGR234*a*. Two copies of NGRIS-4, a 3,316 bp long IS-like sequence which is duplicated in pNGR234*a* (Freiberg et al., 1997) were found on the mega-plasmid and five copies on the chromosome (Perret et al., 1997; 2000). A number of essential genes, such as those required for replication, transcription, translation as well as those linked to primary metabolism were also found on these two replicons (Table 1). As expected of a soil-borne prokaryote, many loci (18%) involved in carbon and nitrogen metabolism were identified (encoding enzymes for the assimilation of nitrate/ammonia, the TCA cycle, or transporter of dicarboxylic acid, etc). Putative symbiotic genes were also found, including mega-plasmid borne loci that are involved in exo-polysaccharide biosynthesis and/or export (Flores *et al.,* 1998).

Putative symbiotic loci which are apparently confined to the chromosome include *fixLJKNOPQGHIS* that encodes an oxidase complex necessary for nitrogen fixation (Flores *et al.,* 1998). Rhizopines are synthesised in *Medicago sativa* nodules inoculated with various *R. meliloti* strains and are thought to give a competitive advantage to the rhizobia which produce them (Murphy and Saints, 1992). Since it was assumed that the genes responsible for rhizopine synthesis (*mos*) and catabolism (*moc*) are confined to *R. meliloti* strains (Rossbach *et al.,* 1995), we were surprised to find homologues of *mocABC,* and *mosA.*

Propagation of rhizobia in the soil, and hence their symbiotic efficiency, probably also depends upon their tolerance to osmotic changes. In this respect, homologues of the *R. meliloti betABC* genes involved in the osmo-regulatory choline-glycine betaine pathway (Pocard *et al.,* 1997) were also found. Other putative chromosomal loci include homologues of the *phbC* and *prsDE* genes of *R. meliloti* which encode a poly-3-hydroxybutyrate synthase (van Slooten *et al.,* 1990) and a type I

Table1. Functional classification of mega-plasmid and chromosomal predicted protein-coding genes of *Rhizobium* sp. NGR234 (V. Viprey, A. Rosenthal, W.J. Broughton and X. Perret, unpublished).

Functional categories	Number
Cell envelope and cellular processes	
Cell wall	17 (1.9)
Transport / binding proteins and lipoproteins	184(20.0)
Sensors (signal transduction)	21 (2.3)
Membrane bioenergetics (electron transport and ATP synthase)	49 (5.3)
Surface polysaccharides biosynthesis and export	25 (2.7)
Sporulation	1 (0.1)
Mobility and chemotaxis	26 (2.8)
Cell division	5 (0.5)
Protein secretion	13 (1.4)
Chaperones / heat-shock proteins	12 (1.3)
Cell killing	8 (0.9)
Intermediary metabolism of :	
Carbohydrates and related molecules	69 (7.5)
Amino acids and related molecules	91 (9.9)
Nucleotides and nucleic acids	11 (1.2)
Lipids	19 (2.1)
Cofactors / prosthetic groups	37 (4.0)
Phosphate	3 (0.3)
Opine-like compounds	8 (0.9)
Sulphur	2 (0.2)
Information pathways	
DNA replication, restriction, modification, repair	26 (2.8)
DNA segregation, recombination and transfer	10 (1.1)
RNA synthesis and modification	19 (2.1)
Protein synthesis and modification	63 (6.8)
Regulatory functions	68 (7.4)
Other categories	
Adaptation to atypical conditions and protection	27 (2.9)
Transposon-related functions	51 (5.5)
Phage-related functions	5 (0.5)
Miscellaneous	52 (5.6)
Total	922 (100)

- Percent putative genes in each functional group is given in brackets.

secretion system (York and Walker, 1997). If PrsD and PrsE of *R. meliloti* are involved in the secretion of enzymes that modify succinoglycans, similar proteins seem to be responsible for the secretion of the nodulation-signalling protein NodO in *R. leguminosarum* bv. *viciae* (Sutton *et al.,* 1996; Finnie *et al.,* 1997). Although it is not clear what role these *prsDE* homologues play in NGR234, it is interesting that such symbiotically active type I secretion systems may exist in other rhizobia.

In conclusion, it seems likely that most essential symbiotic genes are carried on the symbiotic plasmid, pNGR234*a*. Other, perhaps redundant genes involved in nodulation and nitrogen fixation are dispersed over the other plasmid and the chromosome. pNGR234*a* seems to have preferentially acquired IS/MS sequences, suggesting that it is more plastic than the other replicons. Flavonoids and the NodD1 protein exercise primary symbiotic control, but expression of some flavonoid-inducible loci (e.g. the TTSS) is delayed. Finally, the chromosome carries only a few putative symbiotic genes and their role in nodulation remains to be demonstrated.

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THE SYMBIOSIS ISLAND OF A *MESORHIZOBIUM* STRAIN THAT NODULATES *LOTUS*

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1. Introduction

The symbiosis island of *Mesorhizobium* strain ICMP3153 is a 500-kb chromosomally integrated element that transfers to nonsymbiotic mesorhizobia in the environment and converts them to symbionts able to nodulate *Lotus corniculatus* and *L. japonicus.* The island encodes an integrase of the phage P4 family at one end. It integrates into a phe-tRNA gene, reconstructing the gene at the integrase end and producing a 17-bp direct repeat of the 3' end of the gene at the other end (Sullivan, Ronson, 1998). The island was discovered in a study of the genetic diversity of rhizobia nodulating a stand of *L. corniculatus* established seven years earlier in an environment devoid of rhizobia able to nodulate the legume (Sullivan et al., 1995). Although the stand was inoculated with a single *M. loti* strain, ICMP3153, the majority of nodule isolates gave distinct genomic DNA fingerprints. However, when probed with nodulation or nitrogen fixation genes, the nodule isolates and ICMP3153 gave identical restriction fragment patterns. The conserved symbiotic DNA was shown to be part of the chromosome of the strains. Seven strains of nonsymbiotic mesorhizobia that belonged to four genomic species were then isolated from the site where the diverse symbionts were found (Sullivan et al., 1996). The nonsymbionts were all auxotrophic for thiamin and biotin and all but one were auxotrophic for nicotinate. In contrast, ICMP3153 and the diverse symbiotic strains were prototrophic for all three vitamins, suggesting that the transferred element encodes genes required for vitamin synthesis as well as *nod* and *nif/fix* genes. The transfer of the symbiosis island to the nonsymbionts of three genomic species was subsequently demonstrated under laboratory conditions using selection for transfer of vitamin prototrophy (Sullivan, Ronson, 1998).

The term "symbiosis island" was proposed for the element in analogy to the pathogenicity islands found in bacterial pathogens. Like the symbiosis island, many pathogenicity islands are integrated adjacent to tRNA genes, encode an integrase at one end, and convert environmental strains to strains able to form close associations with eukaryotes (Hacker et al., 1997). The symbiosis island differs from pathogenicity islands in that it is larger than any studied to date, comprising about 10% of the genome of its host, and its transmission has been demonstrated (Sullivan, Ronson, 1998).

2. Nucleotide sequence analysis of the symbiosis island

We are in the process of sequencing the entire symbiosis island of strain ICMP3153. This project was undertaken for several reasons. The island is likely to encode most of the genes required to convert a saprophyte to a symbiont; the sequence information should give insight into the transfer mechanism and evolution of islands; and *M. loti* is the natural microsymbiont of *L. japonicus,* a model legume used for studies of the plant contribution to the symbiosis. *M. loti* is relatively uncharacterised but sequence of the symbiosis island will rapidly provide the missing data required to advance genetic studies of both partners. In addition, comparison of the sequence with that of the symbiotic plasmid pNGR234a of *Rhizobium* strain NGR234 (Freiberg et al., 1997) should be particularly informative in identifying new symbiosis-specific genes.

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To date, we have completed 80-100 kb regions at each end of the island and have 3-fold coverage of the remainder of the island. This sequence reveals that the island contains genes which are spread over several replicons in other rhizobia. For example, it contains genes required for microaerophilic respiration and nitrogen fixation (eg. *fixGHIS, fixNOQP*) that are chromosomally located in NGR234. In *Sinorhizobium meliloti thi* genes, *exsBCD* and *dctABD* are located on the second megaplasmid, while in *Rhizobium etli* a *thi* operon is on a nonsymbiotic plasmid (Miranda-Rios et al., 1997). These genes are all located on the symbiosis island. The island also contains several operons found on pNGR234a but not yet implicated in the symbiotic process (eg. *y4tOPQRS* and *y4mKJIH* that both encode ABC transport systems; *y4jJIHGF* that have no other database homologues), operons with similarity to *trb* operons involved in plasmid transfer, several genes which encode metabolic functions including vitamin biosynthesis and glutamate catabolism, and several regions with no database homologues. Unlike pNGR234a, the island does not contain genes required for synthesis of a type III secretion system. However of interest is the finding of genes with strong homology to *virA, virG, virD4* and *virB1-B11* of the Ti plasmid of *Agrobacterium tumefaciens.* In *A. tumefaciens,* these genes encode a type IV pilus required for the transfer of T-DNA to plants. The genes on the symbiosis island include all the genes required to make the pilus and couple the transferred molecule to the transfer apparatus, but none of the genes required for T-DNA processing. Possible roles of the symbiosis island *vir* genes are being investigated.

3. The phe-tRNA gene is also the target of other acquired genetic elements in *M. loti*

Sullivan and Ronson (1998) observed greater sequence variation downstream compared to upstream of the island insertion site among the examined strains. Such variation might reflect additional acquired elements. We have now shown the presence of different genetic elements adjacent to the phe-tRNA gene in the nonsymbionts CJ3 and CJ4. DNA fragments amplified by PCR from downstream of the phe-tRNA gene in each strain hybridised to different subsets of diverse symbionts of the mesorhizobial population. Some strains hybridised to both probes, some to only one or the other, and some to neither, confirming the acquired nature of the DNA. Analysis of partial sequence from the acquired regions showed that one included an integrase gene of the P4 family, suggesting the presence of a new fitness island (Preston et al., 1998), while the other did not contain an integrase but contained genes with strong similarity to the *fhuDB* genes required for hydroxamate siderophore uptake. As hydroxamate siderophores are produced by several fungi and bacteria, the acquired element may give the bacteria an advantage in iron-limited environments.

The symbiosis island may be regarded as a new type of site-specific conjugative transposon. As the tRNA gene is reconstructed following integration, a single strain may acquire multiple fitness islands in a tandem array. Therefore it seems possible that such fitness islands that encode adaptive traits and target tRNA genes may be major drivers of microbial evolution through acquisition.

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DNA REITERATION IN RHIZOBIAL GENOMES: HOW UNUSUAL IS IT?

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Frequent gene reiteration is a hallmark of *Rhizobium* genetic architecture. After its initial discovery in *Rhizobium etli* (Quinto et al. 1982), reiterated genes or genome segments have been a recurrent topic in *Rhizobium* literature. Gene reiteration is a widespread characteristic in *Rhizobium* (Flores et al. 1987; Romero et al. 1997). The unusual accumulation of reiterated sequences in these genomes has inspired a variety of proposed mechanisms for their generation (Romero et al. 1999a). Moreover, the high identity in nucleotide sequence frequently observed among reiterated sequences lead us to identify gene conversion-like events as the mechanism responsible for sequence homogeneity (Rodríguez, Romero, 1998). Besides its particular functional role, homologous recombination between reiterated sequences fosters a variety of genome rearrangements, such as amplifications and deletions (Flores et al. 1988; Romero et al. 1991; Flores et al. 1993; Romero et al. 1995; Mavingui et al. 1998), that occur quite frequently in the *Rhizobium* genome. The genomic plasticity provoked by these rearrangements should have a strong phenotypic impact in some instances, such as nodulation competitiveness (Mavingui et al. 1997; Castillo et al. 1999).

Although widespread gene reiteration and frequent genomic rearrangements are well-documented for rhizobial genomes, other bacteria, such as *Halobacterium* sp. and *Streptomyces* sp. also share these characteristics. Based on the ubiquitous distribution of insertion sequences and the myriad of rearrangements in which they participate, other genomes will surely show significant degrees of genome reiteration and plasticity (Romero, Palacios, 1997). However, a broad overview of the occurrence of these characteristics in the bacterial world is still missing. The current impetus generated by genome sequencing projects is changing the way in which we approach certain biological problems. In the past four years, 21 bacterial genomes were fully sequenced, with 40 more bacterial genomic projects currently under way. The diversity in the bacterial genomic sequences reported, coupled with advances in computer analysis, makes it possible to analyze the degree of gene reiteration in several genomes.

In a recent study (Romero et al. 1999b), we analyzed the bacterial genomes available in the Genome division of the GenBank for the occurrence of identical repeats, using the program Miropeats (Parsons, 1995, available at http://www.ebi.ac.uk/~jparsons/). This program locates identical repeated sequences, larger than a certain threshold, in whole bacterial genomes. Besides a table of analysis, the program generates a graphical display where repeated sequences are joined by arcs, whose height depends on the type of repeat (smaller for direct repeats, taller for inverted repeats). These graphical representations are an ideal way to visualize the extent of reiteration in bacterial genomes. As an example, we show a representation of the distribution of exact repeats (larger than 300 bp) in the sequence of the symbiotic plasmid of *Rhizobium* sp. NGR234 (Figure 1).

The extensive data obtained in these analyses (Romero et al. 1999b), reveal a striking breadth in the abundance of exact reiterated sequences (larger than 300 bp) between bacterial genomes, with *Rhizobium, Helicobacter pylori, Synechocystis, Mycobacterium tuberculosis* and some archaeal genomes being highly reiterated. Interestingly, an *Escherichia coli* strain shows also appreciable reiteration, mainly due to the high copy number of a certain IS (IS5, 25 copies). Raising the

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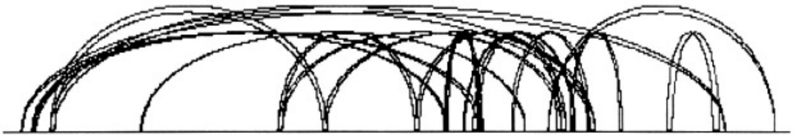

Figure 1. Exact DNA repeats (longer than 300 bp) in the pSym of *Rhizobium* sp. NGR234. The nucleotide sequence of this plasmid was analyzed using the program Miropeats.

threshold of analysis to identify only exact reiterated sequences larger than one Kb produced interesting changes. In this case, the genomes of *Rhizobium, H. pylori, Synechocystis* and *M. tuberculosis* stand out as highly reiterated. Extending this analysis to fully sequenced plasmids larger than 50 Kb confirmed the highly reiterated nature of pNRC100 (190 Kb) in *Halobacterium halobium.* Common between several of these genomes is a high genomic plasticity, provoked by a variety of genome rearrangements. These studies reveal which genomes show a level of reiteration comparable (or even higher!) than the one expected for *Rhizobium* genomes. Current estimations of the level of reiteration in *Rhizobium* are based in random samplings of the genome (Flores et al. 1987) and the sequence of the pSym of *Rhizobium* sp. NGR234 (Freiberg et al. 1997). The results of genomic projects in *Rhizobium* (see this volume) will contribute, among other exciting developments, to clarify the precise extent of reiteration in these genomes.

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THE SYMBIOTIC PLASMID OF *Rhizobium etli*: SEQUENCE, ANALYSIS AND COMPARISON

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1. Introduction

Rhizobiaceae is a family of bacteria able to interact with the roots of higher plants, this family includes several genera of pathogens, i.e. *Agrobacterium* spp., as well as others that can establish a nitrogen-fixing symbiosis with legumes, i.e. *Rhizobium, Sinorhizobium, Bradirhizobium,* etc. *Rhizobiaceae* were originally classified mainly on the basis of phenotypic features such as growth properties, colony morphology and host range (Martínez et al, 1990). Genetic information in these bacteria is usually distributed among various replicons, including the chromosome and several large plasmids.

In most of the *Rhizobium* species, the genes that participate in host range specificity, nodulation and nitrogen fixation processes are located on a single large plasmid. This plasmid is referred to as the symbiotic plasmid (pSym). *Phaseolus vulgaris* L. (common bean) was originally diversified in Meso and South America (Gepts, 1990), and it is an agriculturally important crop. The diversity of *Rhizobium* nodulating *P. vulgaris* has not yet been completely described, but it includes at least three species; *R. etli* (Segovia et al, 1993), *R. tropici* (Martínez-Romero et al, 1991) and *R. leguminosarum* bv. phaseoli (Jordan, 1984). The genetic diversity of the bacteria belonging to *Rhizobium etli* species has been estimated to be very high by the method of multilocus enzyme electrophoresis (Souza et al, 1992). Data obtained by this method strongly correlated with those derived from DNA-DNA hybridization and nucleotide sequence analysis (Milkman, Bridges, 1990; Nelson et al, 1991).

The pSym from *R. etli* strain CFN42T (p42d), a circular structure of 390 Kb (Girard et al, 1991) is conserved only within the plasmids of the species, showing some degree of polymorphism, but overall a high level of gene sequence homology. Moreover, the genetic diversity for the pSym from *R. etli* strains isolated from different geographical origins, so far analyzed, is relatively low.

Two fifths of the p42d includes all the information required to nodulate and fix nitrogen on bean plants (D. Romero, personal communication) and only 5% of the plasmid (18 Kb) either in *Agrobacterium* or *Rhizobium* is necessary to promote nodulation on *P. vulgaris* roots (O. Rodríguez, personal communication). The remaining part of the plasmid, aside from the replicator region, does not participate in symbiosis, but does contain some elements involved in the competitiveness for nodulation. When two of these plasmids (from strains CFN42T and TAL 182) were independently transferred to an *Agrobacterium tumefaciens* plasmidless strain (GMI9023), in order to evaluate their symbiotic performance, no differences were noted in competitiveness nor in nodulation kinetics. Therefore, these plasmids likely contain equivalent symbiotic information, even though the genetic distance between the two chromosomes is relatively high (0.6), (Piñero et al, 1988).

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For how long this plasmid and the *Rhizobium etli* chromosome have been part of the same genome is not known, but since both the genetic distances and the genetic structure for each replicon are different, it is probable that this plasmid has either arrived (via horizontal transfer) or was originated (in relatively recent evolutionary time) in this species, and then it was successfully spread (perhaps by man via bean seeds) to various geographical regions.

2. Current Research

This work describes the progress in the DNA sequence of p42d (90%). We have randomly subcloned, into an M13 vector, the inserts of 11 cosmids that covers the plasmid. The gross sequence analysis allows us to identify the minimal set of genes required for nodulation and nitrogen fixation and the characterization, relative orientation, and comparison of the elements belonging to the different families of reiterated sequences. We have started the refinement, computational analysis, and annotation of the sequence. We are elaborating a gene map, which includes those sequences showing a high conservation with the sequence present in databases. This genetic map has been topologically compared with that derived from the complete sequence of the symbiotic plasmid of the broad host *Rhizobium* strain NGR234, in order to determine the degree of conservation in the physical organization of both replicons. The results show that the structural organization of these elements is not conserved.

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ANALYSIS OF THE SYMBIOTIC GENE REGION OF *BRADYRHIZOBIUM JAPONICUM*

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The physical and genetic map of the *B. japonicum* chromosome revealed that the known nitrogen fixation and nodulation genes are clustered within a region of about 400 kb (Kündig et al. 1993). Because of the complex interactions between the bacterium and the plant, we expected that this chromosomal sector contains additional genes that are involved in the maintenance of an efficient symbiosis. Therefore, we decided to determine its nucleotide sequence. To this end, 410573 bp have been sequenced. The overall G+C nucleotide content is 59.1 %. This is considerably lower than the value we obtained for housekeeping genes at other chromosomal locations (about 65 %). The coding potential was analyzed with the program Glimmer (Salzberg et al. 1998). Accepting AUG, GUG and UUG as start codons, a minimum gene length of 90 bp, and a threshold score of 90 % coding probability, the program identified 937 ORFs (id1-id937) within this region. By raising the threshold score to 99 % coding probability, rejecting ORFs that were overlapping without exhibiting similarity to known genes and increasing the minimum gene length to 150 bp, the number of ORFs was reduced to 393. 34 % of the predicted proteins show similarity to proteins of rhizobia (this does not include IS and related elements). 16 % are similar only to proteins of other bacteria (again IS and related elements are excluded). No database match was found for almost 30 %. Open reading frames that show similarity to genes involved in recombination and integration account for 20 %. About 30 % of the ORFs are related to genes previously identified in the symbiotic plasmid of *Rhizobium* sp. NGR234 (Freiberg et al. 1997).

According to their similarities to database entries and their predicted functions many of the new genes can be grouped.

Most nodulation genes of this strain have already been described. In addition, three ORFs were identified with similarity to *noeI (id808)* and *noeE (id853* and *id855)* of *Rhizobium* sp. NGR234, which are involved in Nod factor biosynthesis (Freiberg et al. 1997 and references therein). However, none of the ORFs was preceded by a *nod* box that is located upstream of most nodulation genes.

The list of the known *nif* and *fix* genes in this organism can be extended by *nifQ, nifW, nifZ* and *fixU*. All genes exist in other nitrogen fixing strains as well. Within the *nif* gene cluster three ferredoxin-like genes were found that could be involved in nitrogenase maturation or activity.

Several ORFs are likely to be involved in the uptake of carbon sources. Id13 exhibits similarity to DctA (55 % identity on the amino acid level to DctA of *R. leguminosarum* gi|231980). A cluster of eight ORFs probably arranged in an operon seems to encode a sugar ABC transporter. Two of the eight genes are encoding a permease. One protein could represent the periplasmic binding component. The ATP-binding protein exhibits highest homology to MtlK (gi|2293417) and MalK (gi|2052275, 52 % identity). The last protein encoded within this operon has similarity to transcriptional regulators. The remaining three ORFs might be involved in the catabolism of the transported substrate.

Because nitrogen fixation requires a molybdenum-containing cofactor it is not surprising that there are two ORFs probably involved in molybdenum uptake. *id17* encodes a putative permease (51 % identity to ModB of *Rhodobacter capsulatm* gi|541288). Upstream of "*modB*" a perfect –24/–12

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promoter region is located as well as a UAS for NifA-binding. The protein encoded by *id147* is similar to ModC of *Rhodobacter capsulatus* (44% identity, gi|585499).

A σ^{54} promoter is also present upstream of the longest ORF *(id930)* comprising almost 10 kb. *id930* probably encodes a peptide synthetase. The sequence, however, does not reveal the composition of the synthesized peptide. Whether this putative peptide serves as a chelator or has a different function remains to be established. The pantetheine cofactor that is needed for peptide synthesis could be synthesized by proteins encoded a few kb upstream of the synthetase. This cluster also includes a regulatory gene and genes probably involved in biotin biosynthesis.

At least one of the ORFs might be involved in the supply of fixed nitrogen to the plant. The product of *id56* is similar to alanine dehydrogenase of several bacterial strains (50-61 % identity). It has been reported recently that alanine is the nitrogen species that is preferentially exported from *B. japonicum* bacteroids in soybean nodules (Waters et al. 1998). This fits well with the observation described earlier by Werner et al. (1980) that alanine dehydrogenase activity is derepressed in nitrogen-fixing *B. japonicum* bacteroids. Thus, *id56* is a good candidate for encoding this enzyme activity. It is also intriguing to note that *id56* is located right next to the nitrogenase genes.

Several of the ORFs encode functions that might have an affect on the interaction with the plant. One large gene cluster that is interrupted by repetitive elements encodes a type III secretion system. It is very similar to that identified in *Rhizobium* sp. NGR234 (Viprey et al. 1998). It was shown earlier, that rhizobia export proteins upon induction with flavonoids (Krishnan, Pueppke 1993). Rhizobitoxine that often induces chlorosis on new leaves of the host plant is another example of a rhizobial product that could interfere with the plant. Although it was reported that *B. japonicum* USDA110 does not produce rhizobitoxine (Minamisawa 1990) the corresponding genes were now identified.

Of high interest to us are also the genes *id568, id636* and *id637*. ID568 has weak similarity to endoglucanase of *Erwinia carotovora* (27% identity, gi|121830), ID636 is similar to polygalacturonase of *Erwinia carotovora* (39 % identity, gi|148472) and the closest related protein to ID637 is pectine methylesterase of *Arabidopsis thaliana* (33 % identity, gi|4567229). Therefore, these "enzymes" could hydrolyze plant cell components.

Furthermore, several putative outer membrane proteins have been identified within this region that could also contribute to the complex interaction of *B. japonicum* and its host plants.

Future work will aim at substantiating all of these predictions.

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MULTIPLE REPLICON IN AZOSPIRILLUM SP.

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The genomic organization in the α-Proteobacteria is highly variable and often very complex. Among the Rhizobiaceae, the *Bradyrhizobium* genome consists of a single circular chromosome of ca. 8Mb whereas in *Rhizobium* species the genetic information is present on combinations of circular chromosomes, 1 or 2 megaplasmids, and small plasmids (Jumas-Bilak et al., 1998). On the other hand, *Agrobacterium tumefaciens,* also a Rhizobiaceae, has two chromosomes, one of which is linear (Allardet-Servent et al, 1998).

Azospirillum sp are diazotrophs associated with important agricultural crops including wheat, maize, banana and pineapple (Baldani et al., 1997; Cruz et al. 1999) and they are classified within the α-subclass of Proteobacteria by 16S rDNA sequence analysis (Young 1992). Seven species have been described within the genus *Azospirillum.* Here we describe the determination of the genomic patterns of intact DNA of strains of three species of *Azospirillum: A. brasilense* (strains Sp7, FP2, Cd and Sp245), *A. lipoferum* (strains Sp59b and JA25) and *A. amazonense* (strains Y2 and Y6). Intact genomic DNA was analyzed using a Pulsed-Field Gel Electrophoresis system (PFGE) to determine the presence and size of replicons. Two different running settings were used to separate molecules of 200Kb to 2 Mb and above 2Mb. All the analyzed strains showed multiple DNA bands with sizes ranging from 0.2 to 2.8 Mb. These PFGE patterns of *Azospirillum* were strain specific.

A. brasilense strains FP2, Cd and Sp7 showed a similar DNA profile with 5 megareplicons each (0.6, 0.7, 0.8, 1.7 and 2.5 Mb) while strain Sp245 also showed 5 replicons however with a clear difference in size (0.7, 0.8, 0.9, 1.8 and 2.6 Mb). The similarity among Fp2, Cd and Sp7 is consistent with them being related to each other, while Sp245 being a different isolate showed a different PFGE profile. *A. lipoferum* strains showed 7 or 8 replicons with sizes ranging from 0.23 to 2.6 Mb and an overall genome size of 7.5 to 7.8 Mb without considering small plasmids. *A. amazonense* showed 4 replicons with sizes varying from 0.7 to 2.8 Mb and an estimated genome size of 7.3 to 7.4 Mb. *A. brasilense* and *A. lipoferum* were indicated to have both circular and linear replicons as determined by PFGE behavior. In *A. brasilense* the three smaller replicons were probable linear DNA molecules while in *A. lipoferum* probably linear replicons were 0.65, 0.71, 0.97 and 1.18 Mb in Sp59b and 0.45, 0.55, 0.85 and 1.1 Mb in strain JA25.

DNA hybridization assays using 16S rDNA as a probe, identified at least 4 replicons of *A. brasilense* and *A. lipoferum* and 2 replicons of *A. amazonense* that hybridized with that gene, which indicates that *Azospirillum sp.* have multiple chromosomes.

DNA hybridization also located the *nifHDK* genes in the 2.5 Mb circular replicon of *A. brasilense* strains Sp7 and FP2. These genes are also located in a circular DNA molecule in the other strains analyzed.

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In summary our results indicate that the *Azospirillum* genus has multiple replicons instead of a single chromosome and that some of those are linear molecules. Also, this is the first report of megaplasmids in *A. amazonense.*

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THE MELILO EU PROJECT: SEQUENCE OF THE 3.7 Mb CHROMOSOME OF *SINORHIZOBIUM MELILOTI*

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1. Introduction

The genome of *S. meliloti* strain 1021 consists of 3 replicons: one chromosome of size 3.7Mb and two so-called megaplasmids of size 1.4 and 1.7 Mb. An international effort is under way aiming at establishing the complete nucleotide sequence of the *S. meliloti* genome (see http://sequence.toulouse.inra.fr/meliloti.html). As part of this effort, the MELILO project, sponsored by the EU and started Nov.1, 1998, has undertaken sequencing the 3.7 Mb chromosome. The organization of the MELILO project is presented together with its current status.

2. Results

2.1 Strategy.

Although shotgun strategies are often favored nowadays, we have chosen a two-step strategy for the MELILO project based on the construction of an ordered chromosomal BAC library, followed by shotgun sequencing of selected BACs. This strategy has two main advantages: it fits the tripartite structure of the genome and it is well suited to multi-site sequencing. A third advantage is that the BAC strategy tremendously simplifies the contigation of sequence data, which is often problematic with large genomes, especially when repeated sequences are frequent as in *S. meliloti.*

2.2 Construction of a chromosomal BAC library.

As a starting point for the MELILO project, four genomic libraries of the entire *S. meliloti* genome were constructed in pBeloBAC11 and pBACe3.6. BACs containing chromosomal inserts were screened by PCR using known chromosomal genes as probes as well as STS generated after subcloning highly purified chromosomal DNA in a pUC vector. 252 chromosomal BAC clones were ordered using a total of 447 markers. An average of 5.5 overlapping BAC clones per marker, correlated with a low rate of deleted or rearranged clones (< 1%) indicate a solid BAC contigation (Capela et al 1999). A subset of 48 clones (mean insert size 80kb) with convenient tiling were selected for sequencing purposes. As an additional benefit to this approach, a high-density physical map (one marker every 9 Kb) of the chromosome was generated that can be used for instance for comparative genomics. In addition, BlastX analysis of STS suggested a possible function for a number of ORFs. All these data are accessible at http://www-recomgen.univ-rennesl.fr/meliloti.

2. 3 Sequencing and sequence annotation.

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Each BAC of the minimal library is sent by the coordinator to one of the laboratories of the consortium for shotgun sequencing. Assembled and publication-quality sequence is sent to the Bioinformatics group in Toulouse that validates and annotates the sequence.

Potential ORFs are predicted using both an extension of the GLIMMER program (Salzberg et al. 1998) and NCBI-BLASTX comparisons against the NCBI-nr database. tRNAs are predicted using the tRNASCAN-SE program (Lowe, Eddy 1997). Each ORF or tRNA-gene is then validated manually. IS and RIME repeated elements previously identified in *S. meliloti* (Osteras et al. 1995, Osteras et al 1998) are mapped.

Annotation uses both automated and manual expert procedures. Validated ORFs are submitted to a set of protein-oriented methods including NCBI-BLASTP (Altschul et al. 1997) against the SP/SPTREMBL (Bairoch, Apweiler 1999) databases as well as ProDom (Corpet et al. 1999) and PROSITE (Bairoch et al. 1997). Transmembrane segments are predicted using TMpred (Hofmann, Stoffel 1993). Gene classification is based on the widely-used EcoCyc database for *E. coli* orthologous genes (Karp et al. 1999) with slight modifications to accomodate *S. meliloti* biological specificities, such as nodulation and nitrogen fixation.

2.4. Overview of sequence data

As of September 1999, 7 BACs have been completely annotated and 25 BACs are being processed. Based on the 675 kb of sequence analyzed so far, the mean GC% is 63%. 642 ORFs were predicted with a mean gene size of 900bp. Intergenic regions are 200 bp-long in average. 91 genes (14%) have no detectable homology to genes present in data bases and 238 (37%) display a partial or global sequence similarity to proteins of unknown function. BLAST access to the data is available at http://sequence.toulouse.inra.fr/meliloti.html

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4. Acknowlegments

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GENOTYPIC CHARACTERIZATION BY PCR DNA FINGERPRINTING AND PARTIAL 16S-23S rDNA INTERGENIC SPACER SEQUENCING OF *Azorhizobiun caulinodans* ORS571

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Azorhizobium caulinodans ORS571, archetypical species of bacteria of Genus *Azorhizobium,* nodulates the tropical waterlogged-adapted legume *Sesbania rostrata* on roots and stem-located root primordia (Dreyfus et al, 1988). Apart from its aerial site of nitrogen fixation, *S. rostrata-Azorhizobium* association is of special interest because nodulation and nitrogen fixation are not repressed by available nitrogen in the soil, stem nodule is very effective in terms of nitrogen fixation and substantial potential for the system as green manure or forage has been referred (Veasey, Valarini, 1997). The azorhizobial stem nodulation, besides the intrinsic importance, can be suitable for searching new niches of nitrogen fixation on plants.The molecular genetic analysis of *the A. caulinodans* ORS571 has been focused on the essential genes for nitrogen fixation *(nif)* although different bacterial loci involved in nodulation *(nod* genes) have been characterized (Van den Eede et al, 1988; Mergaert et al, 1993). We are interested in the study of symbiotic nitrogen fixation genes in *S. rostrata,* specifically those required for stem nodulation. In this report, we have analysed *nif and* rDNA sequences of ORS571 strain, compared to *Rhizobium* isolates, using PCR amplifications with the primers RP01, FGPD807, FGPK492', FGPS1490 and FGPL132', and DNA sequencing of partial 16S-23S rDNA intergenic spacer (IGS) with an Automatic DNA Sequencer. Azorhizobial genomic and plasmid regions were amplified by all the primers. The *Rhizobium* strains isolated from Sesbania root-nodules shared most of their bands discriminating them from the *Azorhizobium,* strain ORS571. The *nif*-primers, considered universal for rhizobial species, were verified to be conserved for azorhizobial strain. Primers FGPD807 and FGPK492', used to amplify parts of *nif* D and *nif* K, are located in most *Rhizobium* strains on symbiotic plasmids. Indeed, plasmid DNA extracted in this work (bands>13kb) provided amplification for both *nif*-primers. DNA fragments of 895bp and 677bp, PCR-amplified and encoding part of 16S-23S rDNA IGS of *A. caulinodans* and *Rhizobium* sp, respectively, were sequenced and retrieved from BLAST Database Search Program, accession n° Z35330 (Kundig et al, 1995) and AJ005010.1. The 16S-23S rDNA IGS nucleotide sequences revealed most significant alignements to *Bradyrhizobium japonicum* rRNA genes and to *Nitrobacter winogradskyi* rRNA genes for the azorhizobial and rhizobial strains, respectively. Stem nodulation assays showed that *A. caulinodans* was stablished as endophyte of *S. rostrata* inducing nodules up to 2m from the inoculation site. A general conclusion from our observations is that rhizobial *nif* and rDNA IGS regions are conserved in *Azorhizobium caulinodans* ORS571 and the IGS nucleotide sequence can belong to *Bradyrhizobium japonicum* rDNA cluster.

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PLASMID-ASSOCIATED TRAITS ARE SPECIES-SPECIFIC

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In order to better understand the genomic organization of bean nodulating strains, we have selected strains isolated from bean nodules of different regions from Spain. These strains were previously classified as *Rhizobium etli, giardinii, fredii,, gallicum* or *leguminosarum* through 16S rDNA and MLEE studies. We have compared them to american *R. etli* strains. The comparison is centered on symbiotic and free-living plasmid-associated traits: plasmid content, localization of *nifH, fixL, fixK* and ***lpsβ*** sequences, and determination of reiterations among them as well as presence of self-transmissible plasmids.

The results obtained suggest that *etli* species is defined by a specific set of plasmid encoded sequences in addition to the 16S rDNA.

We thank Laura Cervantes and Paz Salas for technical help. Supported by grant IN203297 from DGAPA, UNAM, and by the program of international cooperation from CONACYT-CSIC.

SEQUENCING THE 1700 KB PEXO MEGAPLASMID OF *SINORHIZOBIUM MELILOTI. IN VIVO* CLONING OF 60-240 KB FRAGMENTS.

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A genetic map of the pExo megaplasmid, consisting of transposon Tn5-derivative insertions linked in transduction, was previously constructed. To determine the nucleotide sequence of pExo, we wished to clone large regions of this plasmid. Here we describe the development of target constructs consisting of 300 bp from insertion IS50, the Flp recombinase target element, FRT, *oriT* and the *oriV* from RK2 or the F plasmid and a cassette encoding resistance to spectinomycin. We have used this cassette to clone large >50 kb regions of the megaplasmid for subsequent use in shot-gun cloning and sequencing. The fragments are cloned via *ori*T-directed recombination. Employing this strategy we have identified a megaplasmid region which appears to carry the *oriV* of the pExo megaplasmid and is capable of autonomous replication in *Agrobacterium tumefaciens*. Sequencing of this region revealed genes homologous to the plasmid replication proteins RepA, RepB and RepC.

CURING OF THE MEGAPLASMID IN *Rhizobium tropici* STRAIN CIAT899

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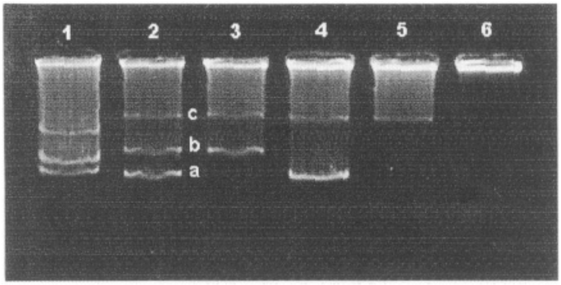
Bacteria of the genus *Rhizobium* generally carry a large amount of plasmids which may represent between 25 to 50% of the total cell genoma (Martinez-Romero et al., 1990). Most of the essential genes required to the symbiotic process are located in a plasmid called pSym. In addition to the pSym, strains of *Rhizobium* often contain other plasmids termed cryptic or non-pSym, whose functions are not well defined. The large number, stability and high molecular weight suggest that cryptic plasmids may play a role in the ecology or physiology of rhizobia. The objective of this study was to construct a library of plasmids cured derivatives from *Rhizobium tropici* strain CIAT899 and determine their functions under symbiotic and free-living conditions. Plasmid profile analysis showed the presence of three plasmids, including a megaplasmid larger than 660 Mda already described by Geniaux et al. (1995). The plasmids were sequentially labelled with the *nptI-sacB-sacR* cassette and cured as described by Hynes et al. (1989) except that the sucrose concentration used in the TY medium was increased to 20% to select the derivatives. Exposure of labelled cells to 44°C resulted in the elimination of the plasmid **a,** while the symbiotic plasmid **b** was cured at 30°C. A derivative carrying only the megaplasmid **c** was obtained from a derivative cured of plasmid **a** when it was submitted to 39 or 42°C. The megaplasmid **c** was eliminated from a derivative cured of both plasmids **a** and **b** at 30°C, resulting in a plasmid-less derivative. This is the first report showing the elimination of a megaplasmid in *Rhizobium* strains. The curing of the megaplasmid may have occurred due the absence of the other plasmids in the cell therefore increasing the probability of *sac* gene insertion.



Plasmid profiles of *Rhizobium tropici* and its plasmid-cured derivatives. Lane 1: referencial strain *Rhizobium leguminosarum* bv. *phaseoli* DB1 (166, 248 and 585 MDa). Lane 2: *Rhizobium tropici* CIAT899. Lane 3: derivative cured of plasmid **a.** Lane 4: derivative cured of plasmid **b.** Lane 5: derivative cured of plasmids **a** and **b.** Lane 6: plasmid-less derivative.

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Acknowledgement

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INTERSPECIES TRANSFER OF NODULATION GENES FROM *BRADYRHIZOBIUM JAPONICUM* HRS STRAINS TO *B. ELKANII*

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HRS strains of *Bradyrhizobium japonicum* possessing high copy number of IS elements, such as RS alpha, RS bata and IS1631 have been isolated from Niigata and Tokachi in Japan. Several lines of evidence indicated that symbiotic regions of HRS strains are subjected to DNA rearrangements. In this work, we demonstrated horizontal transfer of nodulation genes from *B. japonicum* HRS strains to *B. elkanii* USDA94deltaNOD that lacks common nod genes. HRS strains NK5, NC32a and T2 were mixed with USDA94deltaNOD, and placed on HM salt medium. The resultant cell mixture was cultivated and inoculated to *Macroptilium atroperpereum cv. siratro.* As a result, *B. elkanii* cells acquired nod region of HRS strain NK5 and an IS elements, and restored nodulation phenotype. When omega cassette was inserted into the nod region on the chromosome of HRS strain NK5, the interspecies transfer of them *nod* genes was repeatedly detected. We are examining factors and mechanisms that govern the horizontal transfer of nodulation genes.

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NATURAL GENERATION OF NEW SYMBIOTIC PLASMIDS

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We have been interested in the role of plasmids in *Rhizobium etli*. One of the plasmids present in *R. etli* strain CFN42: pCFN42a, is self-transmissible at a high frequency and also functions as a helper for the transfer of the symbiotic plasmid pCFN42d. Through the analysis of transconjugants obtained from conjugation experiments, we have detected the formation of new symbiotic plasmids, which contain segments of the original pSym, and sequences of pCFN42a. Among these novel pSym's there are some which are able to nodulate, transfer at high frequency, gain compatibility with pCFN42d but lose their compatibility with pCFN42a. We think that these plasmids arose through a non- wild type resolution of a cointegrate between pCFN42a and pCFN42d. The generation of such plasmids allows the construction of strains which may now simultaneously contain two pSym's.

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BIODIVERSITY IN RHIZOBIA FROM NATIVE LEGUME TREES FROM URUGUAY

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1. Introduction

Legumes are symbiotically associated with growth-promoting nitrogen-fixing rhizobia and mycorrhizal fungi. Native legumes in Uruguay are studied with the purpose of including them in agricultural or agroforestry systems. The objectives of this work were: 1) to characterize 55 rhizobia isolates from 8 species of native legume trees by their growth characteristics and antibiotic, salinity and acidity resistances; 2) after analyzing results of applications of rep-PCR and FAME techniques, the NTSYS program was applied in order to group the isolates; and 3) to study taxonomic relationships between native isolates and characterized species of rhizobia. Growth characteristics, antibiotic, salinity and acidity resistances of isolates were determined. FAME (fatty acid methyl ester) (Jarvis, Tighe, 1994) and rep-PCR (de Bruijn et al, 1992) techniques were used to characterize this population. The results were analized with the NTSYS-PC program (using simple matching and UPGMA).

2. Results and Discussion

The rhizobia from native legume trees were fast-growing strains that may be included in the *Rhizobium* genus. The generation time varied from 3.0-4.5 h and they produced acid in YEM agar with bromothymol blue. The isolates from *Prosopis, E. crista-galli, E. contortisiliquum* were typical fast-growing rhizobia, and those from *Acacia* grew at an intermediate rate.

Isolates from *S. virgata, P. dubium and L. nitidus* showed more sensitivity to antibiotics. More than 60% isolates were resistant to 50 μg/mL of Ery and 40% to 50μg/mL of Str, but were more susceptible to Rif and Neo. Isolates from different trees showed different behavoir; more than 50% of *Prosopis* and *E. crista-galli* isolates were resistant to more than 50 μg/mL of the tested antibiotics, whereas those from *P. dubium* and *L. nitidus* were more susceptible. Sixty-two percent of strains grew in YEM with 2% NaCl and 16% grew with 3%. All strains grew at pH 5,5; 93% at pH 4,6 and 76% at pH 4,4. The strains from commercial inoculants were more susceptible to these levels of antibiotic, salinity or pH.

The rep-PCR analysis allowed recovery of the strains from nodules after inoculation and showed a great homology between isolates from the same tree and marked differences between isolates from different hosts, in this way showing to be a good tool to follow bacterial strains in natural ecosystems. The characterization of this population by ecological approaches showed 7 groups. *A. caven* isolates showed high homology and the same results were obtained by the rep-PCR determination. Grouping the population with FAME technique made 5 groups. In general, each group contained isolates from the same host. FAME determinations showed to be a very sensitive method for the characterization of a diversity in a microbial populations.

3. Acknowledgements

To Conicyt-BID for support of this work and to the Physical-Chemistry department of the Facultad de Química, Montevideo, for the FAME analysis.

ANALYSIS OF THE *RHIZOBIUM* SP. NGR234 GENOME

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The genome of the broad host-range *Rhizobium* sp. NGR234 is partitioned into three replicons: a 3.2 to 3.5 Mb chromosome, a >2Mb mega-plasmid (pNGR234*b*) and a 536 kb (pNGR234*a*). Although mobilisation experiments confirmed that most symbiotic loci are encoded by pNGR234*a*, sequence as well as hybridisation data showed that chromosomal and megaplasmid loci are also involved in symbiosis.

Analysis of the NGR234 genome started with the construction of canonical ordered cosmid libraries, which led to the full sequence of pNGR234*a*. High resolution transcriptional analyses of all predicted pNGR234*a* genes, helped target new symbiotic loci such as those encoding a type III secretion system. Loci of pNGR234*b* and the chromosome are being studied using shot-gun sequencing combined with transcriptional analyses of selected clones. Plasticity of NGR234 genome was tested, using large DNA repeats as landmarks for predicting rearrangements. Together, these results highlight the dynamic nature of this genome, where horizontal transfer of genes seems to have driven its evolution.

CHARACTERISATION OF A 40-KB REGION OF THE SYMBIOSIS ISLAND OF *MESORHIZOBIUM LOTI* R7A

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The symbiosis island of *Mesorhizobium loti* R7A is a 500-kb, chromosomally-integrated transferable genetic element that encodes most of the genes required for nodulation and nitrogen fixation on *Lotus corniculatus* and *L. japonicus* (Sullivan, Ronson, 1998). The island also contains many other loci including biosynthetic loci for the vitamins biotin, nicotinate and thiamin.

Sequence analysis of a 40-kb region adjacent to the right integration junction of the island revealed several reading frames and operons, including an operon responsible for the biosynthesis of thiamin pyrophosphate. This operon containing the genes *thiCOSGED,* is very similar to that found on the plasmid *pb* of *Rhizobium etli* (Miranda-Rios et al, 1997). These two rhizobial operons have a very different gene organisation from that described for other bacteria such as *E. coli.* Thiamin is formed by the biosynthesis of two precursor moieties (hydroxyethylthiazole monophosphate and hydroxymethylpyrimidine pyrophosphate), followed by the coupling of these moieties to form thiamin monophosphate, which is then phosphorylated to form thiamin pyrophosphate. The rhizobial operons contain structural genes involved in each of the stages of thiamin biosynthesis, but do not contain all of the genes that have been discovered in *E. coli.* They do however contain a novel gene *thiO* (Miranda-Ríos 1997) which may be performing the role(s) of one or more of the absent genes. All of the genes in the *M. loti* R7A *thiCOSGED* operon are involved in thiamin biosynthetic pathway as Tn5 insertional mutations in each of the *thiCOG* and *D* genes yielded strains which are auxotrophic for thiamin. All of the structural genes required for thiamin biosynthesis in *E. coli* have been identified and most have been assigned functions, but no regulatory genes have been identified in any organism (Begley et al., 1999). A LacZ translational fusion to the thiamin promoter region of *M. loti* R7A revealed that, like other thiamin biosynthetic operons, this operon is regulated in response to the presence of thiamin pyrophosphate. The genes responsible for this regulation are present on the chromosome, indicating a coevolution of the mesorhizobial chromosome and the symbiosis island. Similar cases of host chromosomes coevolving with acquired genetic elements are common in bacteria which contain pathogenicity islands.

This region of the symbiosis island also contains an unusual section of~20 kb, which is flanked at each end by partial copies of the insertion sequence IS511. Southern hybridisation showed that this 20-kb region was not present in other mesorhizobia tested. The region contains atypically large reading frames, most of which have a low GC content, and only one of the reading frames (a lysozyme Ml precursor) shows similarity to a bacterial protein. Three of the reading frames (a putative helicase, an S1 nuclease homologue and an ORF with homology to a putative *Arabidopsis* gene family) show significant similarity to proteins that have previously only been described in Eukarya and Archaea. Until now S1 nucleases have only been found in plants and fungi. Therefore it appears that this unusual region was carried into the island as a separate genetic element most likely acquired from a plant or a fungus.

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ANALYSIS OF A 16-KB REGION OF THE *MESORHIZOBIUM* SP STRAIN R7A SYMBIOSIS ISLAND CONTAINING VITAMIN BIOSYNTHETIC LOCI AND A NOVEL FIX REGULATORY GENE

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Genetically diverse symbiotic strains of *Lotus* rhizobia were shown to arise through lateral transfer of a chromosomal element termed a symbiosis island, from a *Mesorhizobium loti* inoculant strain R7A to nonsymbiotic mesorhizobia present in the environment (Sullivan et al., 1995). Subsequently seven strains of nonsymbiotic mesorhizobia comprising four genomic species were isolated from the same area (Sullivan et al., 1996). Characterisation of the nonsymbionts revealed that they were auxotrophic for biotin, nicotinamide and thiamin, whereas the symbionts were vitamin prototrophs. We subsequently isolated a cosmid (p637) from a library of R7A DNA that complemented the nonsymbionts to biotin and nicotinamide prototrophy and mapped to the symbiosis island.

Sequence analysis of part of this cosmid revealed separate *bioBFDAZ* and *nadABC* vitamin synthesis operons, and in addition several genes not previously indicated. Tn*5*-induced mutants in vitamin loci gave the expected auxotrophic phenotypes. Complementation analysis using *E. coli bioBFDA* mutants showed that p637 encoded all the genes required for the conversion of the precursor pimeloyl-CoA to biotin. An insertion in *bioZ,* an *fabH* homologue not previously reported to be required for biotin biosynthesis, gave a Bio$^-$ phenotype. In *E. coli* BioH and BioC catalyse the formation of pimeloyl-CoA. A plasmid expressing BioZ complemented an *E. coli bioH* mutant but not a *bioC* mutant, indicating it plays a role in pimeloyl-CoA synthesis.

The absence of the vitamin loci in nonsymbionts and their presence on the island indicates that they are likely to play an important role in the ecology of the organism. Biotin is a key growth factor in alfalfa rhizosphere colonisation by *S. meliloti* with *de novo* biosynthesis being the most important source (Streit et al., 1996). However our laboratory and field studies showed that biotin biosynthesis by R7A does not contribute to colonisation of *Lotus corniculatus.* One possibility is that the nonsymbionts use vitamin availability as an indicator of the presence of an environment conducive to growth, and hence a signal to resume growth from a stress-resistant state. Plant roots exude a variety of vitamins and the requirement for vitamins in small amounts for a range of metabolic steps makes vitamin availability an excellent indicator of nutrient availability. Vitamin starvation may also be involved in the attainment of a stress-resistant state in the nonsymbionts.

A novel member *fix V* of the *lad* family of transcriptional repressors was also located on p637 and mutants in the gene had a Fix$^-$ phenotype. The majority of LacI family members are involved in the repression of carbohydrate utilisation genes and some such as *B. subtilis* CcpA direct the global flow of carbon in the cell (Nguyen, Saier, 1995). The challenge for the future is to identify a target of FixV. For example one possibility is that FixV represses carbohydrate utilisation operons during symbiosis when organic acids are supplied to the bacteroid.

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GENOMIC ARRANGEMENT OF *NOD* GENE SEQUENCES OF *BRADYRHIZOBIUM* ISOLATES FROM TGX SOYBEAN GENOTYPES IN RELATION TO *BRADYRHIZOBIUM* USDA110

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Soybean nodulates with specific rhizobia and usually requires inoculation with *Bradyrhizobium japonicum* strains. Several varieties (e.g., Malayan and Orba) introduced to Africa (Pulver et al., 1982;) were however adequately nodulated in soils that had no previous inoculation with *B. japonicum* while cultivars from North America were poorly nodulated. The freely nodulating soybean genotypes, termed promiscuous, however, had poor agronomic characteristics. The International Institute of Tropical Agriculture (IITA) selected soybean genotypes that combined the free nodulating traits of the promiscuous genotypes and the improved agronomic characteristics of the North American genotypes. The resultant TGx genotypes, widely cultivated in western and eastern Africa have poor yields due to ineffective nodulation (Okereke and Eaglesham, 1992). This observation led to the suggestion that the TGx soybean genotypes would benefit from *B. japonicum* inoculation. It is therefore important that the genetic relatedness of the indigenous rhizobia nodulating these soybean genotypes to recommended inoculum strains (e.g., *B. japonicum* USDA 110) needs to be elucidated.

A total of 258 *Bradyrhizobium* isolates obtained from TGx genotypes were examined for their symbiotic effectiveness and genetic diversity in relation to *B. japonicum* USDA 110. The genomic arrangement of *nod* gene sequences of *Bradyrhizobium* isolates was examined by PCR protocols. RFLP group analysis was done based on the *Eco*RI- and *Pst*I- restriction digests of the genomic DNAs hybridized with ***nodD***$_2$, *nolA, nodY, nodDYABC, nodS, nodU,* and *nodIJ* gene probes from *B. japonicum* USDA 110 and *nodK* from *B. elkanii* strain USDA 94. The expected proportion of nucleotide substitutions in and around the probed *nod* gene sequences were derived using the equation 6b of Upholt (1977).

The *Bradyrhizobium* isolates were placed into four symbiotic phenotype groups: SymI (41%), ineffective on both soybean genotypes; SymII (20%), effective on both soybean cultivars; SymIII (5%) caused rhizobitoxine-induced chlorosis on the soybean genotypes; and SymIV (33%), effective on TGx soybean genotype but ineffective on N. American soybean cultivar Clark IV. The % divergence of *Bradyrhizobium* isolates from *B. japonicum* USDA110 ranged from 1.8 to >18.1. Clustering did not directly correspond to symbiotic phenotype groups. The results indicate that TGx soybeans are nodulated by diverse *Bradyrhizobium* strains; some strains are closely related to *B. japonicum,* some to *B. elkanii* while others are divergent from conventional soybean bradyrhizobia.

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DEVELOPMENT OF A PHYSICAL MAP AND SEQUENCE TAG CONNECTOR FRAMEWORK FOR THE *BRADYRHIZOBIUM JAPONICUM* GENOME.

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Current microbial genome sequencing programs have largely focused on bacteria of two types, archae and those of importance to human medicine (e.g., *Hemophilus influenzae*). However, agronomically-important bacteria have been largely ignored. *Bradyrhizobium japonicum* is of particular agricultural importance since it is the nitrogen fixing symbiont of soybean, one of the most significant leguminous crops grown worldwide. A majority of humankind depend on this crop as their single major source of dietary protein.

We have initiated a project with the ultimate goal to sequence the entire genome of *B. japonicum* strain USDA110, a total of 8.7 Mbp. We have selected *B. japonicum* strain USDA110 since a physical map has been constructed for this strain via digestion with a single restriction enzyme (Kundig et al., 1993). This strain has also been the major one used for genetic studies. Finally, field and laboratory experiments have shown this strain to be highly competitive for nodulation (Lohrke et al., 1996). Knowledge of the *B. japonicum* genomic sequence would permit study of the various physiological functions performed by this interesting bacterium [e.g., nitrogen fixation, nodulation, denitrification, autotrophic growth on H_2/CO_2, polyaromatic hydrocarbon degradation, secondary metabolism (e.g., polyhydorxybutyrate), respiration under low oxygen, hormone production (e.g., auxin), nickel metabolism, molybdenum metabolism, desiccation tolerance, saprophytic growth in soil, etc.].

As a prelude to a full sequencing project, we have first sought to construct a physical framework for the *Bradyrhizobium japonicum* genome. A bacterial artificial chromosome (BAC) library was constructed from megabase DNA partially restricted with *Hin*dIII. The BAC library contains 4,608 clones with an average insert size of 146 Kbp. This provides about 77 genome equivalents based on an estimated genome size of 8.7 Mbp. A high-resolution physical map of the genome has been constructed with BAC contigs using a fingerprinting approach. Hybridization to the entire BAC library on nylon filters allows rapid mapping of any *B. japonicum* gene with a resolution of a few Kbp, due to the high redundancy of the library. Using this approach, approximately 50 genes have already been mapped. In addition, the ends of 1,152 BAC inserts have been sequenced (forward and reverse). The use of this approach allows the rapid, low-cost development of information rich molecular scaffolds for bacterial genomes. We now propose to use the detailed *B. japonicum* genome scaffold as a tool to direct and finish full-scale shotgun sequencing efforts. The cost of genome sequencing is high. Therefore, this general approach may useful for generating useful genomic information for many bacteria as a starting point for a full sequencing effort.

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SECTION VI

PLANT GENES INVOLVED IN SYMBIOSIS

TOWARDS THE UNDERSTANDING OF THE STRUCTURE AND FUNCTION OF *SYM* GENES

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The progress achieved in the understanding of the structure of nodulins clearly shows that numerous plant genes are involved in the symbiotic plant-microbe interaction. But our knowledge is very limited because the mechanism of action of *sym* genes still remains unclear. In this session, we anticipate new ideas and approaches to uncover the structure and function of *sym* genes and the ways of integration of these two groups of plant genes involved in the symbiotic process.

Very interesting biological questions concerning nodulation are studied by this community. Take for example autoregulation which is presented by A.A.N. Van Brussel from Leiden University. This phenomenon was described many years ago, but now we see the possibility of its explanation at molecular level. Molecular physiology of the transgenic legume plants presented by G. Hernandez from Cuernavaca (Mexico) is a promising way for the elucidation of the nodulin's function using the antisense constructs.

Despite the fact that good progress has been made in these different topics, it is clear that a continuation of this high level research requires a well developed molecular genetic programme on the plant side, which will allow cloning plant genes that play a key role in nodulation process. To achieve this goal, it will be essential to focus efforts on a limited number of host plants, model legumes, for which all the molecular and genetic tools will be developed to facilitate the cloning of key *sym* genes. The importance of the model legumes for molecular genetics of the symbiotic traits was shown by Jens Stougaard with cloning the first *sym* gene (Lunteren).

In this session, several talks around this theme will be presented. Several tens of mutants were obtained in F.J. de Bruijn's group in MSU for *Lotus japonicus.* A good example of the combination of studying "classical" legumes with the model ones will be presented by G.Kiss from Hungary. In this work, a "chromosomal-walking" approach was taken to clone the genes conditioning the ineffective nodulation and the non-nodulation in alfalfa. The work with *sym2* gene in the Agricultural University at Wageningen is also close to the end.

To estimate the amount of work which should be done in this way, it is essential to complete the list of mutants which are known now for the symbiotic traits. According to Due's and our data, the total number of independent mutants in pea is over 200 and among them there are 100 for which complementation analysis is complete. As a result, over 40 pea symbiotic genes were identified (Borisov et al., 1998). The mutations in these genes allow the dissection of the whole process of nodule formation into its elementary steps (Borisov et al., 1998), which are controlled by these genes.

The system of phenotypic codes for classification of nodule developmental stages was originally proposed by Vincent (1980) and then modified by Caetano-Anolles and Gresshoff (1991). We propose to substitute the phenotypic codes, Itf and Bar, by five new ones, Iti, Ith, Itr, Itn, Idd. The final order of the stages identified to date is as follows: Hac - root hair curling, Iti - infection thread

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growth initiation, Ith - infection thread differentiation inside root hair cells, Itr - infection thread differentiation inside root cortical cells, Itn - infection thread differentiation inside nodule tissues, Idd - infection droplet differentiation, Bad - bacteroid differentiation, Nop - nodule persistence.

Numerous described mutants (genes) in pea raise a question about finding key genes, the homologues of which in the model legumes should be the most interesting for cloning. To reveal these key genes, much more data about the biochemistry, physiology, microbiology of the *sym* mutants need to be accumulated before we understand what those “key” genes are. One of the most important characters in symbiosis is the capability of plant to determine the fate of bacteria on the root surface as well as inside the root. Monitoring of the expression of bacterial genes under the influence of plant genes is thought to be the way of revealing the key points (genes) in nodule formation and function. The modern autofluorescent reporter methods developed by H.P. Spaink’s group looked very promising for such an investigation.

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FUNCTIONAL GENOMICS AND GENETIC ANALYSIS OF NODULATION OF SOYBEAN AND *LOTUS JAPONICUS*

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Plant genes control nodulation and nitrogen fixation in all legume symbioses. The need discover the function and structure of these genes, their interplay, their "side-effects" on other morphogenetic steps, and their relationship to other genes involved in the metabolism, and signal transduction and cell division is becoming progressively and feasible in light of recent advances, commonly referred to as functional and structural genomics. Here we demonstrate how map-based cloning as well as promoter trapping have been applied to two legumes, namely soybean and model legume *Lotus japonicus* to discover genes involved in early nodulation responses. The results suggest that the developmental program underlying nodule initiation and pattern control "borrowed" many genes involved in lateral root formation and control.

We focused initially on the supernodulation locus *nts-1* (Kolchinsky et al, 1997) to develop strategies of map-based cloning. In parallel we initiated a large scale, Y-DNA-based transformation of the model legume *Lotus japonicus* (Jiang and Gresshoff, 1997; Handberg and Stougaard, 1992) to trap plant promoters and to possibly obtain flanking sequence as well as knock-out mutants (Stiller et al, 1997; Martirani et al, 1999).

Glycine max (L.) Merr. Variety Bragg was used to produce the supernodulating mutants such as nts382. Mapping of *nts-1* was describe by Kolchinsky et al (1997) and Landau-Ellis et al (1991). Plant were nodulated with *Bradyhizobium japonicum* strain USDA110. BAC construction and positional cloning approaches are described in Pillai et al (1996). A commercially available and micro-arrayed BAC library of *Glycine max* PI437654 was obtained from Clemson University Genome institute. Other BAC clones made from soybean line "Williams" were obtained from the Noble Foundation as well as soybean line "Forrest" from Southern Illinois University.
Lotus japonicus ecotype Gifu was for all transformation experiments (Stiller et al, 1997). Mapping of mutants was done in crosses of Gifu with ecotype Funakura (Jiang et al, submitted). Nodulation was with *Mesorhizobium loti* strain NZP2235 (Jiang et al, 1997)or *Bradyhizobium sp.* NGR234 (Hussai et al, 1999).

Promoter trapping and insertional mutagenesis was described in detail in Martirani et al (1999).

The autoregulation mutants have the interesting characteristic of nitrate tolerance (Carrol et al, 1985). Plants continue to nodulate when exposed to otherwise inhibitory levels of nitrate. Autoregulation mutants like nts382 involve the shoot in the regulatory circuit (Deves et al, 1986). This was determined by reciprocal grafting of mutants shoot onto wild-type roots, resulting in plants with a mutant root phenotype.
We placed *nts-1* close to the pA132 RFLP marker on linkage group H of USDA soybean map (Landau-Ellis et al, 1991). Using our latest mapping analyses *nts-1* is about 0.2-0.4 cM from pUTG132a. *Nts-1* may be as little as 60 kb from pUTG132a.

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Mapping of several nodulation-related EST from a shoot-derived library of nodulated field-grown plants provided two candidate ESTs in the *nts-1* region. Two ESTs, namely Gm036 and GM002, mapped 1.2 and 0.0 cM, respectively, from pUTG132a in the direction of pA381. Gm036 shares high homology to several plant aldolases. PA381 is an amino acid transporter gene.

We used the DNA sequence pUTG132a to construct PCR primers that allowed the isolation of BAC clones from a BAC library constructed with genotype "Williams". Further clones were obtained from an arrayed library (in collaboration with Dr. R. Wing, Clemson University Genome Center, Clemson, SC). These have been confirmed to contain pUTG132a amplifiable regions as well as both endclones of BAC1-N. The Clemson BAC clones, made from cultivar PI437654, were large with an average size of 140kb. We isolated a fast neutron induced mutant of *Glycine soja* with a classical supernodulation phenotype. This mutant lacks pUTG132a and 100kb in the area where the contig of BACs has been constructed. We feel that the combination of deletion mutants, recombinants and BAC contigs with anchoring endclones as well as ESTs for gene discovery.

Promoter trapping and insertion mutagenesis in Lotus japonicus

We have taken advantage of the high tranformation frequencies in *L. japonicus* to explore whether genes involved in nodulation, nitrogen fixation, nodule function as well as lateral root initiation and growth can be discovered. There is substantial evidence that lateral root formation and nodulation are related processes. For example, *har-1* and *nts-1* mutants have an altered root phenotype when inoculated and nodulated. Anatomically, nodulation and lateral root formation both involve the pericycle. Root tip decaptation alters nodulation patterns. Nodules emerge from the same root region, adjacent to xylem poles as do lateral roots. Many nodules in other species are similar to modified lateral roots (e.g. Parasponia, or actinorhizal nodules). While we do not postulate that nodules are lateral roots, we feel that many genes involved in the initiation and growth of lateral roots are utilized in nodulation. To test this hypothesis beyond the physiological and anatomical correlations, we initiated a gene discovery project, utilizing promoter trapping as well as insertional mutagenesis.

The approach utilizes the T-DNA of *Agrobacterium rhizogenes* or *A. tumefaciens.* The binary vector is modified to carry a promoter-less *gus* (beta-glucuronidase) gene on its left side. Upon transfer to the plant genome, most insertion remain inactive, while those inserted into a functional gene become activated. The inserted *gus* gene becomes a histological tag, permitting detailed expression analyses relating to source tissue and physiological responses. The insert also provides a reference piece of DNA from which flanking DNA is isolated by tail-PCR or inverse PCR. Self progeny from transgenic insert plants showing *gus* gene activation should contain 25% homozygous insertions. If the inserted gene is essential for a function, a mutant phenotype could be obtained.

There are two basic approaches for promoter trapping. The first utilizes *A. rhizogenes* and transgenic hairy roots. The approach has the disadvantage of including *rol* genes from *Agrobacterium*, requiring the eventual segregation from these to eliminate possible pleiotropic effects. *L. japonicus* is very susceptible to a range of *Agrobacterium* strains and hairy roots were obtained at a frequency of 90% of inoculated plants (Stiller et al, 1997). Such hairy roots nodulate normally, and express nodulin promoter gene constructs in an expected manner (Martirani et al, 1999). In gene trapping experiments using the promoter-less *gus* gene, expression patterns differ with most independent lines showing expression in the entire root or limited to the cortex and vascular system. Distinct patterns arise, involving lateral root tips, nodules, and vascular regions. Obviously, the tagged genes have composite expression patterns. E believe that such genes may be critical for nodule development but would never have been found by differential expression analysis compared to root tissues. Root sections from selected roots were cultured on antibiotic containing media leading to sterile cultures which were regenerated to primary transformant plants.

Progeny was obtained for further developmental and molecular analysis. We obtained several interesting trapped lines such as MACHINEGUN, PUPILLE, DONUT, BAGEL and ARROWHEAD. MACHINEGUN expressed *gus* in lateral root primordia leading to regular dots along the central vasculature. ARROWHEAD expressed entirely in the root tip region, giving a precise delineation of apical cells. Interestingly, ARROWHEAD did not express in nodule primordia.

The second approach involves hypocotyl transformation of *L. japonicus* with *A. tumefaciens* carrying a binary vector with a promoter-less *gus* gene. The primary transformants are tested for expression, before being selved to obtain stable homozygous insertions. We have isolated tagged lines such as CHEETAH, TIMPA, and FATA MORGANA. In CHEETAH it appears as if *gus* expression is similar to that seen in MACHINEGUN, except that additional foci are noticed in nodule primordia. These become extremely strong within 7 days after inoculation with *Mesorhizobium loti* strain NZP2235, then diminish. In the meantime, lateral root meristems are expressing *gus*independently of the age of the plant and inoculation status. CHEETAH contains 3 copies of the *gus* gene, but only when one of these is active. Segregation analysis suggested near-Mendelian segregation, and perhaps even lethality of the homozygous insertion. We have taken single copy segregants and isolated flanking DNA. We have presently analysing these sequences for promoter activity and putative open reading frames. FATA MORGANA is strongly expressed in the nodule interior as well as some cells of the root pericycle. It appears to be related to leghemoglobin.

The efforts by many over 25 years have opened the possibility of combining biochemistry and physiology. Our task now is to use the best type of mutant for the best application, and to characterize the mutant phenotypes in terms of genomic change, expression at the protein level, and developmental significance. Ironically, old scientific "trades", such as protein purification, enzymology, and histology will become more fashionable. Perhaps most sequencing and clone hunting will be done robotically or at least using"Virtual"technologies.

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A REGION ON THE UPPER ARM OF CHROMOSOME 5 OF *MEDICAGO TRUNCATULA* IS HIGHLY SYNTENIC TO THE *SYM2* REGION OF PEA

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1. Introduction

The long history of pea (*Pisum sativum*) genetics has resulted in many symbiotic mutants. Molecular characterization of the genes involved is important for a better understanding of plant processes involved in the nodulation process. However, none of the genetically identified SYM genes have been cloned, mainly due to the size of the pea genome, which is relatively large ($\pm 4.10^9$ basepairs per haploid genome). A few model legume plant species with a small genome have been selected, namely *Lotus japonicus* and *Medicago truncatula.* For both species a collection of symbiotic mutants will be isolated. However, up to now in pea, there are several mutants available which show a unique phenotype not yet identified either in *L. japonicus* or in *M. truncatula.* As examples of such mutants we like to mention *NOD3* and *SYM2.*

The *nod3* mutation results in a hypernodulating phenotype, which is determined by the root (Postma et al., 1988). This is in contrast to the hypernodulating mutants identified in other leguminous plant species, where the phenotype is always determined by the shoot of the plant. The *SYM2* gene is identified in the pea ecotype Afghanistan. Pea plants harboring the *SYM2* allele of Afghanistan peas *(SYM2-A)* can only be efficiently nodulated when *Rhizobium* leg*uminsarum* bv *viciae* produces Nod factors with an acetyl or fucosyl group at the reducing glucosamine residue (Firmin et al., 1993; Ovtsyna et al., 1998). The *SYM2* gene is especially involved in controlling infection thread growth in the epidermis (Geurts et al., 1997). To facilitate cloning of such genes we raised the question whether it would be possible to use the synteny between pea and *M. truncatula.* Therefore we studied to what extend the genomes of pea and *M. truncatula* are syntenic. Since NODS and SYM2 are located in linkage group I of pea, we focused on this linkage group.

2. Results and Discussion

In addition to *NOD3* and *SYM2*, several other symbiotic genes are located in a region of about 20 cM or in linkage group I; e.g. *SYM5, SYM18* and *SYM19* as well as *Lb*, ENOD7 and ENOD40 (Weeden et al., 1990; Temnykh et al., 1995, Kozik, 1996; Geurts, 1998). Therefore this linkage group was also assigned as symbiotic (sym) cluster (Weeden et al., 1990). Genetic mapping showed that linkage group V of M. truncatula contains several genes which are present in linkage group I of pea; e.g. *EIL3* and *ENOD40.* Furthermore 2 *Lb* clusters are present in this linkage group (D. Cook, unpublished), o study the level of synteny in the region around *SYM2,* we used the pea cDNA marker *W62,* which is tightly linked to the *SYM2* gene, as probe to screen a BAC library of M. truncatula. *W62* encodes a receptor kinase, which is part of a small gene family in pea containing 2 members. The map position of the second member, named H62, is not determined. The identified *M. truncatula* BACs could be assembled in 3 contigs, which were named contig 1, 2 and 3. Hybridization of *W62* to the BACs showed that in contig 1 at least 2 regions with sequences homologous to *W62* are present, whereas in contig 2 and 3 only a single region is present. The homologous sequence present in contig 2 is only weakly hybridizing to *W62* of pea, indicating that it is less homologous. These results demonstrate that in the *M. truncatula* genome 4 *W62*

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homlogous sequences are present whereas in pea only 2 *W62* (like) genes are found.

To determine the map position of the *W62*-like loci in *M. truncatula* we used, in addition to genetic mapping, fluorescence in situ hybridization (FISH) on pachytene chromosomes. This latter method has the advantage that the determined map distances (expressed in µm) might be directly correlated to the physical distance between the loci. Furthermore, by using different BACs from a single contig as probes the orientation of the contig on the chromosome can be determined. To do so, first the pachytene bivalents isolated from pollen mother cells of *M. truncatula* ecotype Jemalong A17 were characterized using the following criteria: relative chromosome length, centromere index, morphology of pericentromeric region, and NOR position. By using BAC clones as probe for FISH we were able to correlate the chromosomes and linkage groups. By using BACs belonging to the 3 contigs as probes, it was determined that they were all located on the long arm of chromosome 5. Contig 1 and 2 are tightly linked (<0.5 µm) and are located between the loci *EIL3* and *Lb-2*; a position comparable to the position of *W62* in pea. Detailed FISH analysis revealed that the average chromatin density of the long arm of chromosome 5 is about 300 kb per µm suggesting that the distance between contig 1 and 2 is less than 150 kb. The third contig is located several µm apart from contig 1 and 2, and is located in between *Lb-2* and *RIP1*.

Synteny-level studies between pea and *M. truncatula* focussed on contig 1 and 2, which were extended by chromosome walking. Within the 2 contigs, genes were identified by random sequencing or using individual BACs as probes to screen a *M. truncatula* root hair cDNA library (Covitz et al, 1998). For precise mapping around the *SYM2* locus of pea, a set of 15 Recombinant Inbred Lines (RILs) was constructed, which genetically dissects the region around *NOD3* and *SYM2*. In each RILs a cross-over event had occurred in the region between the *NOD3/SYM2* flanking markers *PsENOD7* and *OPA-1,* which are about 4 cM apart (Geurts, 1998). So, in this set of RILs on average 4 cross-over events per cM have occurred. The level of micro synteny was determined by RFLP analysis using the set of RILs and the newly identified genes located on the 2 BAC contigs. We were able to map 7 genes. Four of these genes are located on contig 1 and 3 genes on contig 2. All 7 genes appeared to be linked to the *SYM2* locus in pea. Between 2 markers located on one end of contig 1 and *SYM2* a recombination had occurred in 2 RILs. These data demonstrate that there is a high level of synteny between the region around the *SYM2* locus of pea and a region on the upper arm of chromosome 5 of *M. truncatula.* To what extent the gene order in both species is conserved remains to be determined. To study this high resolution FISH on pea pachythene chromosomes could be used. Irrespective of the outcome of such studies it can be concluded that the observed level of synteny provides a solid bases to clone genes of pea (like *SYM2)* by exploiting the high level of synteny between *M. truncatula* and pea.

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PLANT GENES CONTROLLING DEVELOPMENT OF TWO PEA (*Pisum sativum* L.) ENDOSYMBIOTIC SYSTEMS

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1. Introduction

Legume-*Rhizobium* symbiosis is a complex process requiring the expression of numerous symbiotic genes of both partners in an integrated pattern of development. In order to analyse this process analytically it is necessary to use the genetic dissection to identify discrete developmental stages, which are under the control of one or a small group of the genes. For this purpose, wide-range collections of symbiotically defective mutants have been created in different legume species (Phillips, Teuber, 1992). The collection of pea (*Pisum sativum* L.) symbiotic mutants is currently the most numerous. It consists of more than 200 independently obtained mutants which were isolated using 7 different pea genotypes. Many of these mutants have been used for complementation analysis and, as a result, more than 40 symbiotic loci have been identified in pea (Brewin et al., 1993; Due, Sagan, p.c.; Tsyganov et al., 1998). Present study was aimed at a phenotypic analysis of the genetically classified symbiotic mutants of different origin to reveal sequential functioning of the genes identified and to discover biochemical and molecular traits which mark crucial stages in nodule development.

2. Results and Discussion

Sequential functioning of pea symbiotic genes. Phenotypic analysis of a series of mutants has identified 8 discrete nodule development stages (Table 1): Hac, root hair curling; Iti, infection thread growth initiation; Ith, infection thread differentiation inside root hair cells; Itr, infection thread differentiation inside root cortical cells; Itn, infection thread differentiation inside nodule tissues; Idd, infection droplet differentiation; Bad, bacteroid differentiation; and Nop, nodule persistence. Earlier, it was shown that 5 symbiotic genes control the development of endomycorrhiza (Gianinazzi-Pearson, 1996; Kolycheva et al, 1993). Three 3 additional genes, mutation in which decreased mycorrhization, have now been found. All the stages of both symbioses are controlled by the different groups of plant genes determining the fate of the microsymbiont inside the root. Having such a biological "watch" it is possible to analyse other traits of mutant phenotypes to reveal the crucial genes for symbiosis formation and function.

Expression of *dctA*-gene in a series of Fix$^-$ mutants. Fix$^-$ mutants in the genes *sym13, sym31, sym33, sym40* were used. The expression pattern of a *dctA-lacZ* fusion was similar to a constitutive one in all pea lines studied. The expression of this fusion was observed in infection threads and in histological zones II-IV. Increased level of *dctA* expression was found in nodules of pea line carrying mutation in the gene *sym31,* controlling bacteroid differentiation after endocytosis of bacteria. This correlated with the number of recoverable bacteria from the nodules. Thus, it was

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shown that undifferentiated bacteroids have higher activity of the dct-system and it decreases after complete bacteroid differentiation.

Table 1. Pea (*Pisum sativum* L.) symbiotic genes {mutants} controlling {blocked at} various nodule developmental stages.

Hac	**Iti**	**Ith**	**Itr**	**Itn**	**Idd**	**Bad**	**Nop**
sym8	***Sym7***	***sym2***	***Sym5***	***sym33***	***sym40***	***sym31***	***sym13***
sym9	***sym14***	***sym36***	***sym34***		**{RisFixA}**	***sym32***	***sym25***
sym10	***sym35***	***sym38***					***sym26***
sym19	**{K24}**						***sym27***
sym30	**{KN1}**						**{FN1}**
	{KN10}						**{RisFixK}**
							{RisFixN}

Leghaemoglobin biosynthesis and nitrogenase activity in a series of the Fix^- mutants. It was shown that the level of nitrogenase activity in the mutant nodules proves the revealed sequential functioning of the characterised genes. Partial activity was found in the nodules of FN1 early senescent mutant only (Romanov et al., 1996). The level of leghaemoglobin expression appeared in several Fix^- mutants and was highest in the early senescent mutants E135f (*sym13*) and FN1 (Romanov et al., 1996). Detection of legheamoglobin in mutants blocked at earlier stages (mutant genes *sym33* and *sym40* (Tsyganov et al., 1998)) can be explained by their leaky phenotypes. It means that leghaemoglobin cannot be used as molecular marker to prove sequential functioning determined after structural phenotypic analysis.

3. Conclusions

Screening of different mutant traits with respect to nodule structure and function has shown that three of 20 legume plant symbiotic genes should be treated as crucial ones for the determination of the fate of bacteria inside the nodule. The identification of those genes in the model legume symbiotic systems and characterization of them at molecular level looks to be very promising for understanding the molecular mechanisms of their action.

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6. Acknowledgements

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MODULATION OF AMMONIUM ASSIMILATION IN TRANSGENIC LEGUMES DURING THE SYMBIOSIS WITH RHIZOBIUM.

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1. Introduction

In the *Rhizobium* - legume symbiosis the ammonium fixed by the bacteroid is assimilated by the plant, in the nodule cells, through the concerted action of the enzymes glutamine synthetase (GS) and glutamate synthase (NADH-GOGAT). The investigation of our groups on several aspects of this key symbiotic metabolic pathway have included different approaches such as biochemistry, molecular biology and metabolic engineering. The obtaining of engineered legume plants altered in GS or in NADH-GOGAT gene expression – overexpression or antisense inhibition-, specifically in the nodule during the symbiosis with *Rhizobium,* allows to vary quantitatively the flux control of the ammonium assimilation process. The study of such transgenic legumes may lead to a better understanding of the molecular physiology of this metabolic pathway and also, may result in obtaining improved legume germplasm with enhanced efficiency in symbiotic nitrogen fixation.

This work presents our recent advances in the characterization of engineered alfalfa (*Medicago sativa*) plants altered in NADH-GOGAT gene expression and in the establishment of an efficient system for regeneration and genetic transformation of bean (*Phaseolus vulgaris*). Both alfalfa and beans are important agricultural crops in several countries. Alfalfa is the main forage crop in Mexico, it is grown in the central valley where the main dairy production industry is located. Alfalfa plants form indeterminate nodules in the symbiosis with *Sinorhizobium meliloti.* In Mexico bean is the crop with most nourishing, cultural and economic importance. More than a third of the world production of bean comes from Latin America, where it is a basic nourishing crop and constitutes one of the main protein sources for the urban and rural low income populations. Bean plants form determinate nodules with *R. etli.*

2. Results

2.1. Nodule specific modulation of NADH-GOGAT in transgenic alfalfa.

The modulation of NADH-GOGAT expression in the nodule was pursued by obtaining transgenic *Medicago sativa* (Regen SY27X) plants bearing a chimeric gene with the nodule specific soybean leghemoglobin (Lbc3) promoter (Ramlov et al. 1993) and the alfalfa NADH-GOGAT cDNA gene (Gregerson et al. 1993), in the sense or the antisense orientations. Such transgenic plants have been obtained. In this work we present the analysis of some antisense plants, which show the expected phenotype. Ten independent transgenic alfalfa clones bearing the antisense NADH-GOGAT gene were analyzed, as compared to control plants which bear only the marker (*nptII*) gene. The presence of the transgene was confirmed by Southern blot analysis. After 3 weeks of incoculation with *S. meliloti* the NADH-GOGAT activity was assayed both in roots and in nodules of the ten transgenic clones. While the NADH-GOGAT activity in the root of the transgenic plants was similar to that in the control, the activity present in the nodules was lower in 4 of the plants

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analyzed. These four antisense-plants showed from 45 to 70 % NADH-GOGAT specific activity in their nodules, as compared to control plants, and are being characterized molecularly and physiologically. The initial characterization of two of the antisense alfalfa lines named GA89 and GA87, revealed the highest inhibition of nodule NADH-GOGAT activity during different stages of the symbiosis with S. *meliloti*: 35-45% and 50-60% specific activity, respectively, as compared to the activity in nodules of control plants. The inhibition of enzymatic activity coincides with an inhibition of gene expression: the specific mRNA (Northern blot analysis) and protein (Western blot analysis) concentrations in the nodules of GA89 and GA87 plants was diminished. The symbiotic phenotype of these plants was drastically altered: their growth was decreased, at later stages of the symbiosis they look much shorter than control plants. The diminished chlorophyll concentration in GA89 and leaf area in GA87 might indicate that essential physiological processes, i.e. photosynthesis, can be altered as a result of the diminution of the ammonium assimilation metabolic flux through the inhibition of the key enzyme in the pathway.

2.1. Regeneration and genetic transformation of bean (*Phaseolus vulgaris* cv. Negro jamapa)

In order to extend our studies on metabolic engineering to the *Phaseolus vulgaris* – *R. etli* symbiosis we are working on developing an efficient system to obtain transgenic bean plants. In spite of the agronomic importance of bean, an efficient system for regeneration and genetic transformation of beans has not been established. Reports on genetic transformation of bean using particle bombardment showed a very low frequency to obtain transgenic bean plants (Russell et al, 1993). There are some reports that show the susceptibility of bean explants or calli to *Agrobacteium*, but none or very few trangenic plants were regenerated (McClean et al. 1991, Mariotti et al. 1989). In an attempt to establish the genetic transformation of *P. vulgaris* cv. Negro jamapa – the variety that we have used for our research that is widely grown in Mexico- via *A. tumefaciens,* we have developed an efficient in vitro regeneration system. We used cotyledonary nodes as explants, these were incubated in media with BAP (5 mg l^{-1}) for the induction of shoots via direct organogenesis. Around 90% of the explants developed an average of 3 – 4 shoots per explant. An average of 80% from the shoots formed developed roots after incubation in root induction media containing high (50 mg l^{-1}) sucrose concentration. Plantlets adapt well to green house growth conditions, they grow normally and are fertile. We tried to couple the established regeneration system with the genetic transformation via *A. tumefaciens* by cocultivation of the explants with appropriate strains bearing marker (*nptI*I and *gus*) genes. In the initial experiments we used kanamycin in the shoot inducing media for selection of transformed shoot. Though around 90% of the regenerated kanamycin resistant shoots were transformed, shown by PCR amplification of the marker genes, the efficiency for rooting was very low. Eight transgenic bean plants have been regenerated with this protocol, so far; these are fertile and transmit the trangenes to the progeny. We concluded that the rooting capacity of bean shoots was drastically inhibited by kanamycin. We are modifying the protocol, by lowering the time or the concentration of kanamycin for selection and trying other selecting agents, in order to obtain a good efficiency of regeneration of transgenic bean plants.

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AN EFFORT TOWARDS THE MAP BASED CLONING OF A GENE CONDITIONING NON-NODULATION TRAIT IN TETRAPLOID *MEDICAGO SATIVA*

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1. Introduction

The MN-1008 mutant conditioning non-nodulation phenotype was isolated from a tetraploid *Medicago sativa* cultivar by Peterson and Barnes (1981). This mutant displayed no root hair deformation, no root hair curling or cortical cell division (Dudley and Long, 1989), however variability in membrane depolarisation after Nod factor treatment was detected (Felle et al., 1995). In addition, this mutant was resistant to vesicular-arbuscular mycorrhiza (Brudbury et al., 1991) but spontaneous nodules appear occasionally on the roots (Caetano-Annolés et al., 1993). From early segregation studies it was proposed that two unlinked recessive mutations were responsible for this phenotype (Peterson and Barnes, 1981). According to the above properties it is thought that in this mutant the perception of the rhizobial signal molecules or the first steps of their subsequent signal transduction pathway are impaired. Consequently, this mutant may be an important key to understand some of the early events in nodule formation elicited by rhizobial Nod factors.

2. Results and Conclusions

In order to isolate the *nod* gene first we had to determine its location on the linkage map of alfalfa. For mapping this trait, tetraploid F2 populations segregating the non-nodulation phenotype were produced after crossing the MN-1008 plant with a nodulating *M. sativa* cultivar (cv. Nagyszénási). Self-mating of the resulted Fl plants generated the segregating F2 populations. Selecting appropriate individuals and based on the Bulked Segregant Analysis (Michelmore et al., 1991) we identified RAPD markers co-segregating with the mutation. With the help of the diploid mapping population these RAPD markers were mapped on linkage group five (LG5) of the *Medicago* genetic map (Endre et al., 1997). This region spanned more than 10 cM genetic distance on LG5, therefore more molecular markers had to be searched for in order to saturate the region and find the more tightly linked markers as starting points for the chromosomal walking experiments. The diploid mapping population and the Bulked Segregant Analysis were used to look for additional molecular (mostly RAPD) markers between RFLP markers U71 and U492 in LG5 (Kaló et al., 1999), where the non-nodulation mutation linked to. Testing more than 500 different 10-mer oligonucleotide primers in PCR experiments resulted in 43 new RAPD markers mapped in this region. Some of these selected markers were mapped (map transfer) in the tetraploid Nod-segregating populations.

At the same time two of the tetraploid F2 segregating populations (progenies of NAB and NBW F1 plants) were extended in order to determine the segregation ratio of the non-nodulation phenotype as well as the recombination frequencies in the region more precisely. The plant test and the segregation data allowed us to conclude that (i) many plants with low viability died before reaching maturity; (ii) the evaluation of the nodulation phenotype of the plants 6 weeks after the infection gives ambiguous results, therefore the non-nod characters should be tested later as well; and (iii) the segregation ratios of the non-nod phenotype (detected non-nod plants in the surviving population) in different F2 populations are closer to the theoretical segregation ratio characteristic for a single gene mutation.

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For fine mapping of the *nod* gene, the F2 segregating population of the NAB F1 plant has been chosen (with more than 2500 individuals) and selected markers from the diploid mapping population were tested. The first aim of the genotyping work was to detect polymorphism for all of the four alleles, namely the two from the non-nodulating parent NM-1008 and two from the wild type *M. sativa* plant, respectively. For this purpose different techniques were used, RFLP hybridizations, specific PCR amplifications (SCARs from RAPDs) run in polyacrylamide gels (besides agarose), and SSCP. Several markers for which genotype could be determined for the appropriate alleles were used to screen all individuals of the NAB F2 population and plants carrying recombinant chromosome for this region were selected for further analyses. Besides ordering the molecular markers the non-nodulating phenotype could be mapped more precisely with the increased number of individuals in the population. Having tested over 2000 plants for recombination and finding markers tightly linked to the *nod* gene, isolation of BAC clones and the physical mapping were started.

For physical mapping and chromosomal walking experiments the BAC library of *M. truncatula* (kindly provided by Dr. D. R. Cook; Texas A & M University, College Station, USA) was used (Nam et al., 1999). Before starting the recombinant DNA work however, the microsynteny in this region between *M. sativa* and *M. truncatula* was tested using some *M. saliva* markers. Mapping these markers in *M. truncatula* it was demonstrated that the location and order of these markers were the same in both plant species. Markers proved to be tightly linked to the *nod* gene were used as hybridization probes to screen the library filters. Positive clones were identified and analyzed further. The isolated end-fragments of the BAC clones allowed us to identify overlapping clones and thus to build a contig in the region. The resulted contig spans about 600 kb. With the help of molecular markers for which we have both genotype data and their locations in the BACs determined the actual size of the region carrying the *nod* gene could be delimited at a region of about 400 kb. We are in the process of sequencing this contig which may lead to identify candidate gene. This gene than can be used to complement the mutation in transformation experiment. We also consider the possibility that no candidate gene will be found. In this case systematic complementation experiments can help to identify the *nod* gene.

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GENETIC LINKAGE MAPPING AND LOCATION OF GENOMIC REGIONS ASSOCIATED WITH NODULATION AND BACTERIAL DISEASES IN _PHASEOLUS VULGARIS_*L

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RFLP- and RAPD-based maps in recombinant inbred populations have been useful to identify major genes in common bean *(Phaseolus vulgaris* L.) for resistance to anthracnose (Adam-Blondon et al. 1994 a, b; Young and Kelly 1997), bean common mosaic virus (Haley et al. 1994; Melotto et al. 1996; Johnson et al. 1997), bean golden mosaic virus (Urrea et al. 1996), rust (Miklas et al. 1993; Haley et al. 1993; Jung et al. 1996) and common bacterial blight (Nodari et al. 1993 a, b; Jung et al. 1996, 1997; Yu et al. 1998). Several traits such as number of days for maturity, number of days for flowering, seed dormancy, harvest index, node number (main stem), pod number, photoperiod sensitivity, pod length and seed weight are already available since the publication of the classical linkage map of common bean (Bassett 1991). Attempts to construct high density linkage maps in a single mapping population, by the joining of several linkage maps developed by the University of California-Davis (Nodari et al., 1993a; Gepts et al. 1993), the University of Florida (Vallejos et al. 1992; Boutin et al. 1995; Yu et al. 1998) and University of Paris-Orsay (Adam-Blondon et al. 1994 b) has being established in common bean, as other plant species, thus enabling to detect common genes among varieties (Freyre et al., 1998).

We have determined QTLs for nodulation and bacterial diseases in the bean cross Bat-93 x Jalo EEP-558 population consisting of recombinant inbred lines ($\mathbf{F_9}$), derived from the $\mathbf{F_2}$ population in which the original Davis map was obtained. The core map included 470 RFLP, 570 RAPD markers and some 40 biochemical and/or morphological markers. QTLs for nodulation by Rhizobium tropici strain CIAT-899 (NN), resistance to common bacterial blight (CB) caused by Xanthomonas axonopodis pv. phaseoli (Xap) or var. fuscans (Xaf), wildfire caused by Pseudomonas syringae pv. tabaci (Pst) and Agrobacterium tumor caused by strain Chry5 were identified. Eight markers distributed within three linkage groups ($\mathbf{B_6}$, $\mathbf{B_7}$ and $\mathbf{B_{10}}$) were shown to be associated with nodule number; $\mathbf{B_7}$ with strong correlation with CB (Xap,Xaf) and SID and $\mathbf{B_{10}}$ with wildfire and Agrobacterium

The majority of the QTLs associated with second internode length - SID ($\mathbf{B_5}$, $\mathbf{B_7}$, $\mathbf{B_8}$ and $\mathbf{B_9}$) was associated with CB resistance (Xap, Xaf), indicating linkage between these traits. Analyses of Agrobacterium tumors were also made in the parentals (BAT-93 - susceptible and Jalo EEP558 - resistant to strain Chry5) and the progenies, mapping QTL loci in $\mathbf{B_1}$, $\mathbf{B_2}$, $\mathbf{B_3}$, $\mathbf{B_9}$,$\mathbf{B_{10}}$ and $\mathbf{B_{11}}$. Wildfire (Pst) was mapped in $\mathbf{B_2}$, $\mathbf{B_3}$, $\mathbf{B_7}$, $\mathbf{B_9}$ and $\mathbf{B_{10}}$. QTLs for bacterial diseases found in $\mathbf{B_2}$, $\mathbf{B_7}$ and $\mathbf{B_9}$ were found in tight associations, suggesting the existence of gene clusters involved in the control of these bacterioses, or either the same genes or pleotropic effects are involved in the expression of these phenotypes and

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Rhizobium nodulation may be associated with the domestication syndrome, co-evolving with the host from a pathogenesis status to the symbiotic association.

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TWO SUBCLASSES OF YELLOW LUPIN PR10 PROTEINS AND THEIR POSSIBLE FUNCTION DURING THE SYMBIOSIS DEVELOPMENT

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1. Introduction

Intracellular pathogenesis-related proteins of PR10 class are ubiquitous in the plant kingdom: their homologues have been found in various species belonging to both dicotyledonous and monocotyledonous plants (Walter et al., 1990). Many common allergens present in birch pollen grains (Breiteneder et al., 1989), celery (Breiteneder et al., 1995) and apple (Vanek-Krebitz et al., 1995) are also included in this group. The PR10 proteins are small (16 - 18 kD), slightly acidic and resistant to proteases (van Loon et al., 1994).
Some of the PR10 proteins have been identified after pathogen invasion, wounding and other environmental stress conditions, which suggests their involvement in the general plant defence mechanism. However, the physiological function and any contribution of PR10 proteins to the defence mechanism remain unknown. High amino acid sequence homology and the similarity of the expression pattern to that of ginseng ribonuclease indicate that PR10 proteins RNase activity may be involved in the defence reaction which has led to their classification as ribonuclease-like PR proteins (Moiseyev et al., 1997). The birch pollen allergen *Betv*1 shows RNase activity *in vitro* (Bufe at al., 1996; Svoboda et al., 1996). There are also suggestions that PR10 proteins play an important role in plant development since they have been identified in dry seeds (Warner et al., 1994; Barrat, Clark, 1993). Constitutive expression was observed in roots (Crowell et al., 1992; Sikorski et al., 1996, 1999; Myloma et al., 1994), stems (Warner et al., 1994) or flowers (Breiteneder et al., 1989; Warner et al., 1993, 1994; Constabel and Brisson, 1995; Huang et al., 1997; Swoboda et al., 1994). Senescent leaves also often have elevated levels of PR10 protein (Crowell et al., 1992). Some PR10 homologues have appeared to be induced by phytohormones like cytokinin (Carpin et al., 1998), abscisic acid (Itturiaga et al., 1994) or ethephon, an ethylene releasing compound (Broderick et al., 1997).

In yellow lupin we have identified two gene families encoding two PR10 proteins subclasses, 1PR10.1 (156 AA) (Sikorski et al., 1999) and L1PR10.2 (158 AA), and we summarise all their properties in this paper.

2. Materials and Methods

The cDNA family encoding a new subclass of yellow lupin PR10 proteins composed of 158 amino acid residues (L1PR10.2) was identified during screening of cDNA library of *Bradyrhizobium* sp. *(Lupinus)* inoculated plants using *Medicago truncatula* N13 cDNA (MtN13) (Gamas et al., 1998) as a probe. RNA was isolated from lupine roots at the different stages of plant development.

Northern blot analysis of PPR10.2 gene transcripts in developing roots of non infected and *Bradyrhizobium.* sp. *(Lupinus)* inoculated plants was performed using a ^{32}P-labeled coding sequence of L1PR10.2B cDNA in 50% Formamide, 5x SSC, 0.5% SDS, 5x Denhardt solution, 5% Dextran sulphate, 100 mg/ml Herring sperm DNA at 42°C. The blot was finally washed in

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the presence of 0.1x SSC/% SDS at 68°C. The LlPR10.2 cDNA probe was specifically hybridised to PR10.2 encoding RNAs. There was no cross-hybridisation to LlPR10.1 cDNAs.

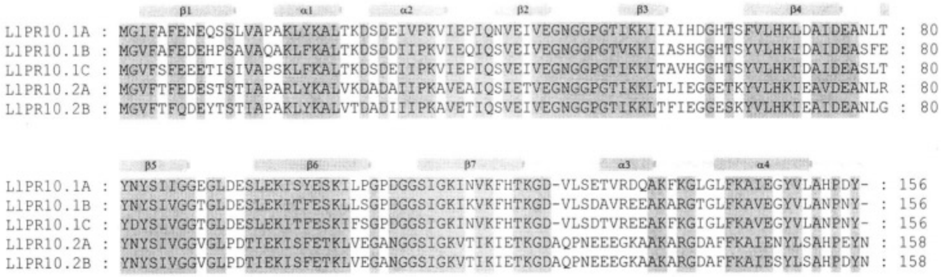
3. Results and Discussion

Multigene families encode proteins of PR10 class in yellow lupin. We have identified two homologous genes classified as LlPR10.1A and LlPR10.1B coding for the first subclass of yellow lupin PR10 proteins. Screening of the B. sp. (Lupinus) infected yellow lupin cDNA library with MtN13 cDNA as a probe did not lead to the identification of any homologue closely related to MtN13, induced during nodulation. Instead, numerously represented genes encoding a new PR10 subclass were found. We named the proteins belonging to the second subclass as LlPR10.2A and L1PR10.2B. We also identified a third gene coding for a protein homologous to the PR10.1 subclass proteins - LlPR10.1C.

The yellow lupin PR10 subclasses have different protein length: the LlPR10.1 protein homologues consist of 156 and LlPR10.2 - 158 residues. The identity between members of the first subclass is 76-85% (91-95% similarity) at the amino acid level. In the second subclass we observed 89-91% identity (94-96% similarity). There is 57 - 59% identity (76 - 78% similarity) between the PR10 protein subclasses. The amino acid sequence alignment of all yellow lupine PR10 proteins (Fig.1) clearly shows that they reveal several common conservative regions. In consequence, the predicted secondary structures (seven β-sheets and four α-helixes) are almost identical.

The most conservative region among all known PR10 proteins is glycine-rich loop GxGGxGxxK (residues 46- 54), called P-loop (Saraste et al., 1990), similar to motifs present in nucleotide-binding proteins. It might be implicated in the RN-ase activity of the PR10 proteins (Moiseyev et al., 1997;Biesiadka et al., 1999).

The PR10 coding sequences for two protein subclasses vary in translation stop codon (TAG in PR10.1 and TAA in PR10.2 genes) and polyadenylation signal. All PR10 genes posses one intron at the same position (after the first letter of codon 61), but the length of the intron is different in every gene (Sikorski et al., 1998).



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L1PR10.1A : MGIFAFENEQSSLVAPAKLYKALTKDSDEIVPKVIEPIQNVEIVEGNGGPGTIKKIIAIHDGHTSFVLHKLDAIDEANLT : 80
L1PR10.1B : MGVFAFEDEHPSAVAQAKLFKALTKDSDDIIPKVIEQIQSVEIVEGNGGPGTVKKIIASHGGHTSYVLHKIDAIDEASFE : 80
L1PR10.1C : MGVFSFEEETISIVAPSKLFKALTKDSDEIIPKVIEPIQSVEIVEGNGGPGTIKKITAVHGGHTSYVLHKIDAIDEASLT : 80
L1PR10.2A : MGVFTFEDESTSTIAPARLYKALVKDADAIIPKAVEAIQSIETVEGNGGPGTIKKLTLIEGGETKYVLHKIEAVDEANLR : 80
L1PR10.2B : MGVFTFQDEYTSTIAPAKLYKALVTDADIIIPKAVETIQSVEIVEGNGGPGTIKKLTFIEGGESKYVLHKIEAIDEANLG : 80

L1PR10.1A : YNYSIIGGEGLDESLEKISYESKILPGPDGGSIGKINVKFHTKGD-VLSETVRDQAKFKGLGLFKAIEGYVLAHPDY-  : 156
L1PR10.1B : YNYSIVGGTGLDESLEKITFESKLLSGPDGGSIGKIKVKFHTKGD-VLSDAVREEAKARGTGLFKAVEGYVLANPNY-  : 156
L1PR10.1C : YDYSIVGGTGLDESLEKITFESKIFSGPDGGSIGKINVKFHTKGD-VLSDTVREEAKFKGIGLFKAVEGYVLANPNY-  : 156
L1PR10.2A : YNYSIVGGVGLPDTIEKISFETKLVEGANGGSIGKVTIKIETKGDAQPNEEEGKAAKARGDAFFKAIENYLSAHPEYN : 158
L1PR10.2B : YNYSIVGGVGLPDTIEKISFETKLVEGANGGSIGKVTIKIETKGDAQPNEEEGKAAKARGDAFFKAIESYLSAHPDYN : 158
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Figure 1. Alignment of amino acid sequences of *Lupinus luteus* PR10 class proteins. Secondary structure is common for both PR10.1 and PR10.2 subclasses. The nucleotide sequences of full-length cDNA clones coding for yellow lupin PR10 proteins were registered in EMBL GeneBank under the following accession numbers: Llpr10.1a - X79974; Llpr10.1b - X79975; Llpr10.1c - AF180941; Llpr10.2a - AF170091; Llpr10.2b - AF170092.

3.1. Expression pattern of LlPR10.1 protein subclass

LlPR10.1 proteins are differentially expressed in various plant tissues. The LlPR10.1B is constitutively expressed in the whole plant, whereas the LlPR10.1A is present in root and stem but absent from leaf, petiole and pollen grains. Both proteins are down-regulated during nodule development, however the high level of both homologues is observed in nodule cortex of mature and senescent nodule. The LlPR10.1 proteins are entirely absent from bacteroid tissue.
Leaves treatment with either symbiotic or pathogenic bacteria (*B.* sp. (*Lupinus*) and *P. syringae,* respectively) caused the induction of LlPR10.1A protein. The level of L1PR10.1B expression seemed to be slightly increased in the course of infection. The mechanical stress (leaf wounding) did not trigger the expression of protein 1A and did not change the level of protein 1B.

3.2. Expression pattern of LlPR10.2 protein subclass

Despite of high nucleotide sequence homology of all cDNAs encoding lupine PR10 proteins we were able to distinguish between transcription pattern of both PR10 protein subclasses at high stringency conditions (0.1x SSC at 68°C) using ^{32}P-labeled coding region of PR10.2B gene as a probe (Fig. 2). Northern hybridisation analysis revealed transient expression of LlPR10.2 genes in 1-day old lupin germs and down-regulation during nodule development in *Bradyrhizobium* sp. (*Lupinus*) infected roots. Comparison of the Northern hybridisation for both PR10 protein subclasses shows similarities in the expression pattern during symbiotic interactions, however genes of the second subclass are suppressed earlier (starting from 9 dpi) and appear at low level in senescent nodule (36 dpi).

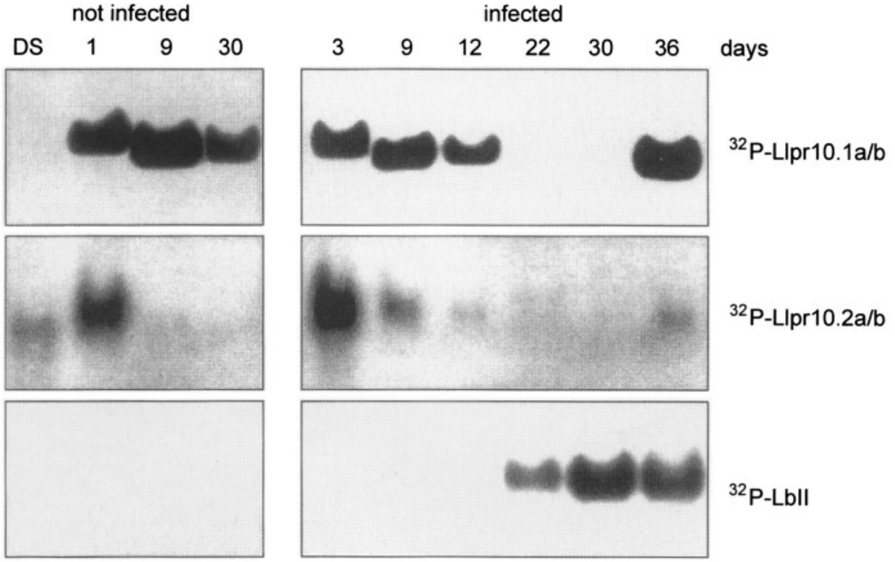

Figure 2. Northern analysis of LlPR10 gene transcripts. ^{32}P-labeled coding fragment of LlPR10.1A, LlPR10.2B and LbII cDNA clones were used as probes. Comparative leghemoglobine hybridisation was used as a marker of nodulation.

The data presented here are consistent with the proposed function of PR10 proteins in general plant defence mechanism. In the plant-microbe interactions, which are beneficial to the plant host (symbiosis), the expression of defence proteins should be suppressed to enable symbiosis development. On the other hand, presence of these proteins in the early stages of symbiosis may be a part of the mechanism by which the plant controls infection and, therefore, regulates nodulation (feedback control of nodulation). The existence of different PR10 gene subfamilies, a number of homologous proteins and their biological activities remain still an intriguing question. The differences in the expression pattern among yellow lupin PR10 proteins suggest their variable functions in plant defence reactions and developmental programme.

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EARLY NODULINS AS MARKERS OF VARIOUS DEVELOPMENTAL STAGES DURING NODULATION OF *MEDICAGO TRUNCATULA* BY *SINORHIZOBIUM MELILOTI*

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1. Introduction

Nodulation is a complex process that can be dissected following various strategies. The identification and characterisation of nodulin genes can provide valuable tools to analyse different stages of nodulation, particularly to study the signals involved in their regulation and to explore the biological functions which need to be turned on.

2. Results

We have undertaken a comparative analysis of a series of *Medicago truncatula* early nodulin genes that we previously identified by different approaches (*MtN1-30:* Gamas et al., 1996; *MtAnnl:* de Carvalho Niebel et al., 1998). A combination of tools has been used to characterise the regulation pattern of several of these genes: in situ hybridization on nodules and roots, study of response to purified molecules (Nod factors and phytohormones), and analysis of transcriptional activation by several *Sinorhizobium meliloti* mutants (*exoA, nodA, nodH* and *nodFL*). These mutants are quite useful because they are able to differentially affect various plant symbiotic responses, such as epiderm and inner cortex activation, nodule organogenesis, preinfection-thread formation, and infection (Ardourel et al., 1994 ; Timmers et al., 1999).

These studies have allowed us to distinguish five regulation classes among early nodulin genes. The first two comprise genes which are activated early during nodulation, and also at later stages. Class I genes, such as *MtN5, MtAnn1, MtENOD11, MtENOD12* and *MtENOD40,* can be efficiently induced by purified Nod factors, but also by a *nodFL* mutant of *S. meliloti,* which produces altered Nod factors. Class II contains nodulin genes, such as *MtN6* (Mathis et al., 1999), which show no induction in response to the *nodFL* mutant. These genes are thus activated by a program requiring w.t. Nod factors to be triggered, in contrast to class I genes which can be activated by modified Nod factors, possibly through the non stringent receptor for Nod factors, postulated by Ardourel et al. (1994), and designated as "signalling Nod factor receptor". RT-PCR analyses show that MtN6 seems to be activated by purified Nod factors at much lower level than class I genes. In response to *S. meliloti, MtN6* is induced, as early as one day post-inoculation, at very defined locations in the outer root cortex (Mathis et al., 1999), in association with cells containing structures likely to be preinfection threads (van Brussel et al., 1992). This differs markedly from the pattern observed at the same time point for *MtENOD11* or *MtENOD12,* which are first transcribed in all epidermal cells of the nodulation-competent region (Pichon et al., 1992). In nodules it is not possible to distinguish the class I and II genes on the basis of their tissue specificity. Thus most of them are expressed in the so-called preinfection zone, located between the apical meristem and the growing infection threads.

A third class of *M. truncatula* early nodulin genes strictly requires bacterial infection to be transcriptionally activated, since they are not induced by an *exoA* mutant of *S. meliloti.* We observed by in situ hybridization that transcripts of one such gene, *MtN*9, appear about two days post-inoculation, only in cells containing infection threads, whether in root hairs or root cortical cells. In mature nodules, *MtN9* transcripts are found in the proximal part of the infection zone, again in close association with infection threads.

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A fourth class contains nodulin genes, such as *MtN13* (Gamas et al., 1998), which are transcribed somewhat later (from about three days post-inoculation), specifically in cells of the nodule outer cortex, suggesting that they need the nodule meristem to form before being induced. However they do not need *S. meliloti* infection, in contrast to a fifth class which corresponds to genes turned on at a similar time (thus later than class III), but strictly requiring bacterial infection.

In addition to regulation studies it is important to explore the biological importance, as well as the mode of action of early nodulins. We have studied in more details *MtN6,* which displays homology to proteins found in several organisms, among which one, the protein FluG from *Emericella nidulans,* has been characterised at the functional level. Interestingly FluG plays a key role in controlling the organogenesis of conidiophores (Lee, Adams, 1994), through the synthesis of a small diffusible signal yet to be identified. *MtN6* is homologous to the N-terminal half of FluG, but we have been able to find, by heterologous screening, cDNA clones representing different homologues (called *MtGSI:* see accompanying poster by Mathis et al.) to the missing C-terminal domain, which is homologous to the GSI class of glutamine synthetases, so far only described in prokaryotes. *MtGSI* genes are expressed constitutively in the root and none of them appears to be upregulated during nodulation. One attractive hypothesis is that *MtN6* plays its role during nodulation by interacting physically or functionally with the *MtGSI* domain(s) (see recent support for this type of hypothesis in Marcotte et al., 1999). It is quite possible that *MtGSI* proteins have evolved from a glutamine synthetase activity to another biochemical function, possibly regulated or modified by *MtN6* during nodulation.

Our strategy of marker identification/characterisation is now being extended with a program of functional genomics (coordinators: P. Gamas and E. Journet), aiming at analysing more comprehensively the *M. truncatula* transcriptome associated with two types of symbiotic interactions, namely with *S. meliloti* and with endomycorrhizal fungi. This project is based upon a collaboration between our laboratory, V. Gianinazzi-Pearson's group (INRA Dijon), and the Genoscope (Evry, France). A collection of 15 000 clones from three cDNA libraries (representing respectively non-inoculated root tips, 4-day-old *S. meliloti*-induced root nodules and *Glomus mossae-M. truncatula* mycorrhizae) is being sequenced, which will allow us to generate a large EST data base and new tools for future studies.

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EARLY SYMBIOTIC FUNCTIONS OF *SESBANIA ROSTRATA*

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Symbiosis on the semi-aquatic tropical legume *Sesbania rostrata* presents several special features. In the case of nitrogen shortage, *Sesbania rostrata* plants will, upon inoculation with the microsymbiont *Azorhizobium caulinodans,* nodulate on both root and stem. The nodulation takes place at predetermined positions, namely at lateral root bases on the root system and at the basis of dormant adventitious root primordia on the stem. Upon submergence in water, these primordia may grow out to form adventitious roots. So, basically on *Sesbania rostrata* lateral root base nodules are formed. Nodulation via the root hair pathway has not been observed, although root hairs are present on the root system, also under conditions of waterlogging. Nodule initiation is very similar on root- or stem-located sites and strictly dependent on bacterial Nod factor signaling. Nodule development is hybrid. In the early stages, an indeterminate nodule originates from a primordium induced in the inner-mid cortex. The young nodule will mature into either a round determinate nodule without meristem or into an elongated indeterminate nodule with a persistent apical meristem. On the stem, only determinate nodules have been observed. On the root system however, both types can occur depending on environmental conditions, the choice being mediated by ethylene (Fernández-López et al. 1998). Intercellular infection is the primary, normal invasion pathway on this host. The primary intercellular entry is an active process and, just like the induction of the nodule primordium, dependent on Nod factors (D'Haeze et al. 1998, and this volume).

We have made use of the great number and easily accessible stem-located predetermined nodulation sites and the synchronous development upon simultaneous inoculation to study the early plant responses of nodule initiation. On the one hand, several *S. rostrata* cDNA clones were isolated based on homology to available sequences. On the other hand, differential display was used to compare RNA pools of primordia before and several days or hours after inoculation with *A. caulinodam* (Goormachtig et al. 1997; Lievens et al. in preparation). The present collection of *S. rostrata* symbiotic genes, confirmed by Northern analysis, comprises two clones without homology to known sequences as earliest induced transcripts (2 hours after inoculation). Several spatial transcript accumulation patterns were determined by *in situ* hybridization. The patterns were very diverse and corresponded to induction of gene expression in peripheral cells of the adventitious root primordium (an acidic class III chitinase Srchi24), in the cortex, and later the nodule primordia (mitotic cyclin, histone H4, ENOD40, extensin, chalcone reductase), in the developing nodule parenchyma (ENOD2, chalcone reductase, acidic class III chitinase Srchi13, ENOD40), in the developing central tissue (GA-20-oxidase, extensin, Srchi13), and associated with bacterial infection (peroxidase, gibberellin-20-oxidase, pectin methyl esterase, chalcone reductase, Srchi13). The symbiotic genes were correlated with cell division, production of secondary signals (for organ development and/or infection), or were related to genes that have been characterized mostly in the context of defense, wound, or ripening response.

More extensive data are available for the two acidic class III chitinases, Srchi24 and Srchi13. Recombinant proteins were expressed in *Escherichia coli* and partially purified to raise polyclonal antibodies. Immunolocalization showed similar protein patterns as those of the RNA transcripts. Enzymatic assays to study biochemical activities revealed that Srchi13 could degrade both Nod factors (Goormachtig et al. 1998) and pentameric chitin substrates. For Srchi24 however, no activity could be

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detected. This might be due to an amino acid replacement in the putative catalytic domain. Sense and antisense constructs are currently being studied in transgenic hairy roots induced on *S. rostrata* upon *Agrobacterium rhizogenes* infection. The possibility that Srchi24 may bind, but not degrade Nod f factors is being investigated. As for the GA-20-oxidase, the enzymatic activity of which has been demonstrated in collaboration with Dr. P. Hedden (Long Ashton, UK), a similar approach of functional knockout is being followed. Intriguingly, the GA-20-oxidase transcripts accumulate very early, in cells located ahead of the infecting bacteria.

Our future experiments will look for secondary signals involved both in organ development initiation and in bacterial entry. Intercellular bacterial entry in *S. rostrata* is dependent on Nod factors and strong indications for a role of H_2O_2 and ethylene have been obtained (see D'Haeze et al. this volume). Although limited, our data support the hypothesis that nodulation makes use of the recruitment of cell division and defense tools and that the symbiotic bacteria evolved to produce bacterial signals that function as moderate fungal chitin elicitors, leaving open the possibility that they also may mimic plant oligosaccharide signals.

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CHARACTERIZATION AND ANALYSIS OF SYMBIOTIC GENES IN ALFALFA AND WHITE SWEETCLOVER

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1. Introduction

The symbioses that exist between legumes and Gram-negative bacteria of the family Rhizobiaceae and also with arbuscular mycorrhizal fungi share common features. For example, *MsENOD40* and *MsENOD2* are early nodulin (*ENOD*) genes that are expressed not only during alfalfa (*Medicago sativa* L.) nodule organogenesis, but also in mycorrhizae (van Rhijn et al. 1997). The involvement of symbiotically-expressed genes other than *ENOD* (van Rhijn et al. 1997, Albrecht et al. 1998) and leghemoglobin (Fr,hling et al. 1997) genes in the two plant-microbe interactions is unknown. However, recently, Balestrini et al. (1999) have shown that the lectin gene PsNlec1 is expressed in pea mycorrhizae.

We have cloned three “seed” lectin genes from alfalfa and are investigating their involvement in symbiotic associations as well as in other aspects of plant development. Some details about *MsLEC1* and *MsLEC2* were published earlier (Brill et al. 1995; Hirsch et al. 1995). Studies on the site of *MsLEC1* gene expression are still in progress. Information about the expression patterns of *MsLEC2* is presented here.

We are also developing molecular tools for analyzing white sweetclover (*Melilotus alba* Desr.) because as a small, self-autogamous diploid, it is more genetically tractable than alfalfa (Hirsch et al. in press). Also, like alfalfa, white sweetclover is nodulated by the well-studied and genetically well-characterized, nitrogen-fixing symbiont, Sinorhizobium meliloti.

2. Materials and Methods

2.1. Cloning and characterization of *MsLEC2*. A (EMBL3 Sp6/T7 genomic DNA library of *M. sativa* (L.) cv. Chief (Clontech Laboratories Inc., Palo Alto, CA) was screened with an *MtLEC1* from *Medicago truncatula* bv. Jemalong (Bauchrowitz et al., 1992) probe as described previously (Brill et al. 1995). Two overlapping genomic DNA fragments were identified and sequenced [Genbank Y16754].

2.2. RNA transfer blot analysis. RNA was isolated from various tissues of alfalfa, using the RNA Stat-60 total RNA isolation reagent kit according to the manufacturer's protocol. Total RNA was subjected to electrophoresis in a 1% formaldehyde-agarose gel and blotted onto Nytran. The probes were prepared as described in Hirsch et al. (1995). A probe made from *Msc27* (Kapros et al. 1992) was used as an internal control to standardize loading. After hybridization, the blots were washed at 65° C with 0.1X SSPE and 0.5% SDS and exposed to film for at least 56 h.

2.3. Genomic Southern blots were performed according to standard procedures (Sambrook et al. 1989). Ten μg of DNA were loaded per lane after digestion with *Eco*RI, *Bam*HI, and *Hind*III.

3. Results and Discussion

3.1. Alfalfa. Previously, we determined that MsLEC2 was expressed at low levels in roots and stems, but at very high levels in flowers (Hirsch et al. 1995). On some northern blots, we had detected *MsLEC2* expression in nodules, but not consistently.

A more detailed analysis confirmed the high level of expression in flowers as well as lower expression in both uninoculated and inoculated roots and also in developing nodules (data not shown). On the other hand, *MsLEC2* expression tapered off in mature, nitrogen-fixing nodules. These results are in contrast to *MtLEC2* expression for *M. truncatula*. *MtLEC2* appears to be a

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pseudogene based on the presence of a two-nucleotide frameshift mutation in the coding region (Bauchrowitz et al. 1992). Nevertheless, transgenic *M. varia* plants carrying an *MtLEC2* promoter-*gusA* fusion showed GUS activity in mature nitrogen-fixing nodules only and not in nodule primordia (Bauchrowitz et al. 1996). Also, Bauchrowitz et al. (1996) did not describe *MtLEC2* expression for organs other than nodules. Figure 1 demonstrates the increase in *MsLEC2* expression in flowers that occurs with developmental age, and the decrease in mature flowers. The numbers refer to a staging system, from stage 1 to stage 8, assigned to alfalfa flowers as they develop (R.E. Satterthwaite, unpublished).

We have also investigated the spatial expression patterns of *MsLEC2* transcripts in roots, nodules and flowers. *MsLEC2* transcripts were localized to nodule and root apical meristems as well as to flower petals, among other floral structures (data not shown).

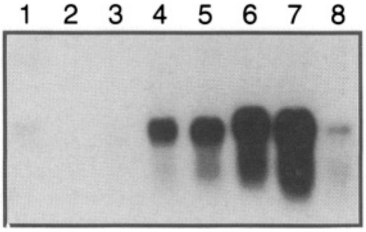

Figure 1. Northern blot of total RNA isolated from alfalfa flowers at different stages of development.

3.2. Sweetclover. Using an MaLEC PCR fragment (W.M. Karlowski, unpublished) as a probe, we detected a single, strongly hybridizing band to sweetclover (Melilotus alba) DNA on a genomic Southern blot. Thus, sweetclover appears to have only a single lectin gene, making it a much better system to determine the involvement of lectins in symbiotic interactions. We are currently characterizing this gene further.

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NIN, A DEVELOPMENTAL REGULATOR OF ROOT NODULE INITIATION IN *LOTUS JAPONICUS*

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The first genetically defined symbiotic locus required for module *in*ception (*Nin*) has been isolated and characterized from a *Lotus japonicus* mutant tagged by an *Ac* transposable element. Following inoculation with *Mesorhizobium loti* strains nodules are not developed on the *nin* mutants (Schauser et al., 1999). Phenotypic characterization shows that the organogenic process is arrested early and that the invasion of the root tissue is not initiated. The *nin* mutant allele segregates genetically as a monogenic recessive but due to the continued activity of the autonomous *Ac* element, both stable mutants and stable revertants could be identified. At the original integration site, these excision events results in small sequence rearrangements, known as footprints. The *Ac* footprints, detected in the isolated allelic series of stable mutants and stable revertants with wt phenotype, confirmed that an *Ac* insertion caused the *nin* mutation. Footprints introducing frame shifts were found in stable mutants while stable revertants carried sequence insertions maintaining the *Nin* gene reading frame. Detailed phenotypic characterization of stable mutants showed that the initial lipochitin-oligosaccharide signal perception and the recognition of the *M. loti* microsymbiont was at least partly functional in the *nin* mutants. Formation of infection threads and cortical cell division was not observed. A role for the *Nin* gene in the signal transduction or cellular activation processes preceding root nodule inception was predicted from these observations. The structure of the NIN protein shows similarity to transcription-factors and indicate a function as transcriptional activator of downstream genes required for initiation of root nodule development. A dimerization domain of heptad leucine repeats close to a basic region and putative activation domains are the characteristic features found. Conservation of this basic heptad repeat region between the NIN protein, the Mid protein from *Chlamydomonas* and several *Arabidopsis* proteins defines a novel putative DNA binding/dimerization domain. Two transmembrane segments were suggested by several protein structure prediction algorithms and the activity of the NIN protein may therefore be posttranslational regulated by membrane binding. This was previously found for the human SREBP sterol regulators and the *Drosophila* Notch transmembrane receptor. The working model for the functional role of the *Nin* gene hypothesises the presence of membrane bound NIN protein in the appropriate target cells. Bacterial recognition followed by signal perception-transduction would then initiate proteolytic cleavage and relocalization of the NIN carboxy terminal transcriptional regulator peptide to the cell nucleus. Subsequent transcriptional activation of *Nin* in dividing primordia cells are involved in activation of downstream genes required for establishing the nodule primordia. In this model a secondary *Nin* dependent signal would promote infection thread growth and negatively regulate root hair competence. Absence of this secondary signal leads to the lack of infection thread formation, excessive root hair response and expansion of the infection zone observed on the *nin* mutant plants. In order to test this model ongoing experiments are directed towards localization of the NIN protein and the predicted cleavage products. The postulated DNA binding activity is investigated and the downstream response genes approached.

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EXPLORING THE GENETIC POTENTIAL OF RICE FOR FORMING SYMBIOTIC ASSOCIATIONS WITH RHIZOBIA

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Leguminous plants are capable of forming symbiotic association with rhizobia. Interaction between the legume host and its bacterial partner leads to the formation of a specialized plant organ, the nodule, in which rhizobia carry out nitrogen fixation. In order to formulate strategies for developing rice-rhizobia symbioses, adopting a systematic approach, we initially studied the extent of genetic predisposition of rice to form an intimate association with rhizobia. For rhizobia to infect the legume root, the bacterial *nod* genes must be induced by plant-produced flavonoids. Our studies indicated that the roots of specific rice cultivars exude compounds that are able to induce, to a low extent, the transcription of the *nod* genes of *Rhizobium* sp. NGR234 and *B. japonicum* USDA110 (Reddy et al. 1999a). These results suggest that rice root exudate either contains very low concentrations of the appropriate activators of *nod* gene expression and/or includes antagonistic substance(s) that inhibit(s) activation of the rhizobial *nod* genes. Neither rhizobia nor purified NodNGR factors, however, could elicit root hair deformation or cortical cell divisions leading to true nodule development in rice. A primary mode of rhizobial invasion of rice roots is through epidermal cracks and fissures created during emergence of lateral roots. Infection was be *nod*-gene independent and does not involve the formation of infection threads (Reddy et al. 1997). Moreover, rhizobial invasion provoked a mild defense response localized to the colonization site.

During legume nodulation, specific plant genes, the early nodulin (*ENOD*) genes, that are required for normal development of nodules, are induced. If rice can be engineered to interact with rhizobia, the presence of many of the proteins known to be required for legume nodule development will be required. Recently, we showed that cDNAs of several legume *ENOD* genes hybridized to DNA from a wide variety of rice genotypes indicating that homologues of *ENOD* genes are widespread in rice (Reddy et al. 1999b). Characterization of two of these *ENOD* homologues, *OsENOD40a* (Kouchi et al. 1999) and *OsENOD93a* (Reddy et al. 1998a), from rice revealed ORFs encoding peptides having considerable homologies to *ENOD40* and *ENOD93.* Recently, an EST deposited in the DNA databank (DDBJ) revealed the a second *ENOD40* homologue (= *OsENOD40b)* in rice with a distinct expression pattern from *OsENOD40a* (H. Kouchi, unpublished). *ENOD40* has been proposed as playing a pivotal role in nodule formation in legumes. In alfalfa, transient expression of *ENOD40* driven by 35S CaMV promoter was found to provoke division of cortical cells with net amyloplast accumulation, suggesting a critical role for *ENOD40* in the promotion of processes leading to the development of nodule primordium (Charon et al. 1997). In rice, over-expression of 35S-*MtENOD40* resulted in major alterations in growth and development, and the plants could not be maintained. Microscopic analysis of the roots of these transgenic rice plants revealed several anatomical changes such as inhibition of the formation of cortical schlerenchymatous layer and occasional differentiation of bifurcated vascular bundles in a newly developing lateral root primordium. The transgenic plants also exhibited morphological abnormalities like the production of highly deformed root hairs and sporadic development of hypertrophies from lateral root primordia. In addition, it is intriguing that in rice roots, over-expression of *MtENOD40* led to extensive deposition of amyloplasts in cortical cells particularly around the stele. To ascertain if regulation mechanisms related to the activation of the *ENOD40* expression are shared between rice and legumes, we developed transgenic rice carrying the *GmENOD40(2)* promoter fused to GUS,

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and analyzed the activity of the promoter in rice. Similar to legumes (Kouchi et al. 1999), in rice, the expression of *GmENOD40(2)* promoter was confined to vascular traces of the root and stem, though the induction of *GmENOD40(2)* is greater in the vascular bundle at the sites of lateral root emergence than in the vascular trace away from it. Kouchi et al. (1999) found that the expression of rice *ENOD40* in soybean roots was also confined to the vascular tissue of roots as well as nodules, thus evidencing that the pattern of expression was essentially the same as soybean *ENOD40.* These findings indicate that legume and rice *ENOD40*s share similar regulatory mechanism(s) and confirm our results with the *MtENOD12* promoter (see below) that the signal transduction machinery essential for legume *ENOD* gene expression is conserved, at least partially, in rice.

The demonstration that Nod factors are perceived by rice would be a significant step toward devising strategies to make rice amenable to nodulation. To determine whether Nod factors are recognized by rice, we introduced the Pr*MtENOD12*-GUS in rice and analyzed its responsiveness to NodNGR factors (Reddy et al. 1998b). The results of the study showed that Nod factors are able to activate the *MtENOD12* promoter in rice root cortical cells, endodermis, pericycle and parenchymatous cells in stele under nitrogen-limited conditions. Werthmuller et al. (1998) showed that the expression of *MsENOD12* in transgenic rice roots is enhanced by the application of Nod factors in the presence of auxin. These findings imply that rice has a mechanism to perceive Nod factors, and also possesses a signal transduction system to enable subsequent activation of the legume *ENOD* gene promoters. However, rice root hairs appear to be recalcitrant to NodNGR factors as neither deformation (Reddy et al. 1997) nor activation of the *MtENOD12* promoter was elicited in them in response to Nod factors (Reddy et al. 1998b). The apparent inability of epidermal cells to respond to Nod factors may be due to the absence of an appropriate cell receptor(s) and/or the absence/repression of Nod signal transduction in these cells.

In legume-*Rhizobium* symbiosis, the host provides the genetic information for the development of nodules. The role of the rhizobia is to trigger the host-plant genes to facilitate infection and help create a nodular-niche for nitrogen fixation. It is unlikely that a monocot, such as rice, possesses the complement of genes or genetic programs involved in nodule ontogeny that could be induced by rhizobia. However, a reason for optimism is that, although rice does not develop a symbiotic association with rhizobia, it does enter into symbiotic associations with mycorrhizal fungi (Khan, Belik 1995). Genetic links between the processes involved in nodulation and arbuscular mycorrhiza have been found in legumes (Harrison 1999). Thus, rice may possess part of the genetic program necessary for entering into endosymbiotic associations with rhizobia (Reddy et al. 1997).

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BIOLOGICAL NITROGEN FIXATION CAN SUSTAIN YIELD IN *PHASEOLUS VULGARIS L.*[*]

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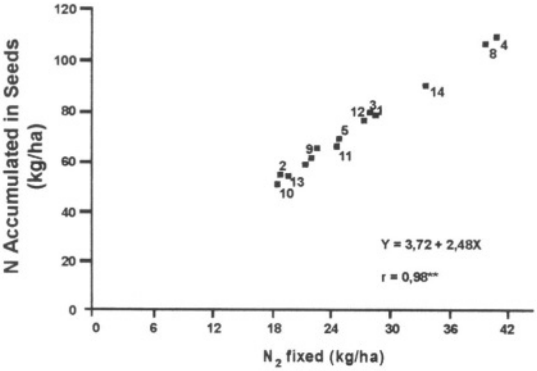
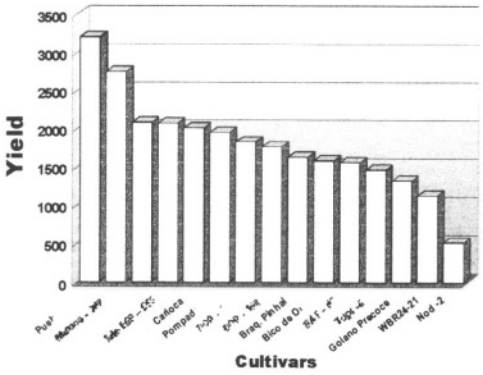
Biological nitrogen fixation (BNF) in common beans was evaluated in 15 varieties, grown in lysimeters with ^{15}N labelled organic-N. Three growth stages were evaluated - R5 (nodulation), R7 (N_2 fixation) and R9 (N_2 fixation and yield). Genotypes Puebla-152 and Mexico-309 were superior in BNF, supporting an average of 40 kg N ha^{-1} from BNF with high yields - 3200 kg and 2750 kg ha^{-1}, respectively. Nitrogen derived from air (%NDA) was not a good parameter to distinguish genotypic differences for BNF ability at both R_7 (40-46.8%) and R_9 (36.3 and 39%) stages. Using the ^{15}N-soil method, where no exogenous ^{15}N-fertilizer was added into the lysimeters, a wide genotypic variation was detected and correlated with their high potential to accumulate fixed-N_2 and to produce more grain (r = 0.98; P<0.01). Differential abilities to translocate the fixed-N_2 from shoot and pods to seed were also observed among the varieties.





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MOLYBDENUM AS COMPONENT FOR N_2-FIXATION AND YIELD ENHANCEMENT IN BEAN VARIETIES

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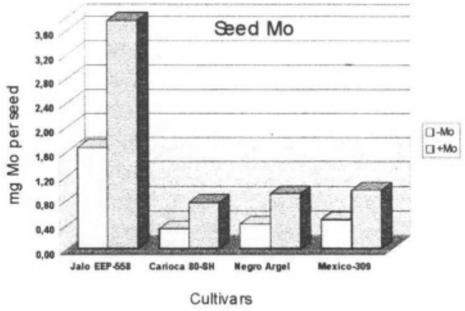
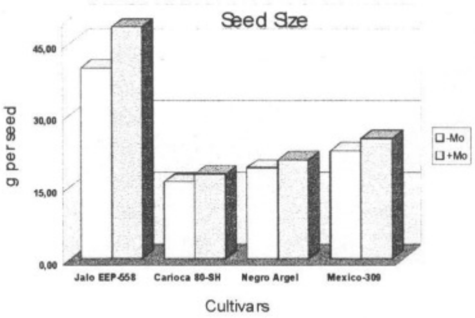
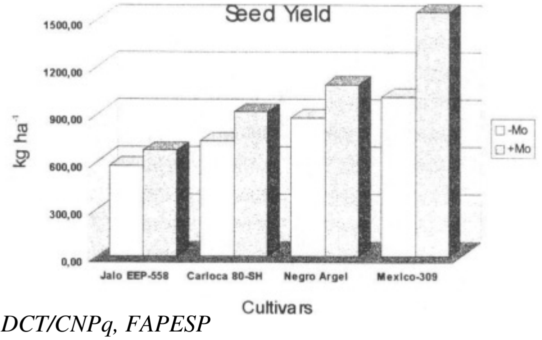
Molybdenum can enhance nitrogen fixation and N assimilation from soil/fertilizer in bean plants, thus may enhance grain yield. The seasonal variation of nitrate reductase (NR) and nitrogenase (acetylene reduction activity-ARA) activities was evaluated in four common bean varieties - two commercial (Jalo EEP-558 and Carioca 80-SH) and two high N_2-fixing lines (Mexico-309 and Negro Argel), grown in lysimeters with ^{15}N-labelled organic N. Ammonium molybdate at two levels (0 and $45\ g\ ha^{-1}$) was sprayed on bean leaves at 21, 28 and 35 days after emergence (DAE). At mid-flowering (42 DAE) the commercial varieties Carioca 80-SH and Jalo EEP-558 presented lower ARA rates than Mexico-309 and Negro Argel (5.5, 9.2, 15.3 and $17.3\ \mu mol\ C_2H_4\ plant^{-1}\ h^{-1}$, respectively). A better response to Mo was observed among the elite N_2-fixing varieties, with increments in nodule number, plant dry matter and N accumulation and yield components, including Mo in the seeds. Grain yield increases from 1013 to $1546\ kg\ ha^{-1}$ (53%) in Mexico-309, 878 to $1092\ kg\ ha^{-1}$ (24%) in Negro Argel, 733 to $916\ kg\ ha^{-1}$ (24%) in Carioca 80-SH, and 576 to $673\ kg\ ha^{-1}$ (17%) in Jalo EEP-558 were obtained when Mo was added. In all varieties, the amount of biologically fixed nitrogen was significantly increased in response to Mo. The amounts of fixed nitrogen were 44, 31, 27 and $15\ kg\ N_{fixed}\ ha^{-1}$ for Mexico-309, Negro Argel, Carioca and Jalo EEP-558, respectively..







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USE OF AUTOFLUORESCENT REPORTERS IN STUDYING INTERACTION BETWEEN RHIZOBIA AND LEGUMES

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Recently, genes came available encoding autofluorescent proteins (AFP) with different spectral properties. Examples are GFP (green), CFP (cyan) and YFP (yellow). These proteins are being used as visual markers and they make it possible to monitor processes, such as infection, *in vivo.* Plasmids expressing constitutively GFP, CFP or YFP were constructed and crossed into several rhizobia strains. By using confocal microscopy with different filtersets it was shown that it is feasible to identify individual rhizobia strains in a mixed bacterial culture and at different steps during nodulation. It was even observed that two differently-labeled strains may occupy the same nodule.

Naturally occuring autofluorescence in plants makes it sometimes hard to use AFPs as a reporter depending on the conditions. Therefore a chimaeric gene consisting of *gusA,* encoding β-glucuronidase (GUS), and *gfp* was made. This bifunctional reporter gene combines the advantages of both GUS (high sensitivity) and GFP (*in vivo* detection) and it is now being used as a marker gene in transgenic *Lotus japonicus* plants to test plant responses upon inoculation with rhizobia and Nod factors.

FINE MAPPING OF THE NON-NODULATION TRAIT IN ALFALFA

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The tetraploid MnNC-1008(NN) *Medicago sativa* mutant is defective in early plant responses, that is neither root hair curling nor cortical cell division occurs after bacterial infection, and therefore is thought to be impaired in the perception of rhizobial signal molecules or in the first steps of their subsequent signal transduction pathway. A tetraploid F2 population segregating the non-nodulation phenotype was used to identify RAPD markers co-segregating with the mutation. We have mapped these RAPD markers on linkage group (LG) five of the diploid *Medicago* genetic map. This part of the LG spanned more than 10 cM genetic distance therefore more molecular markers had to be searched in order to saturate the region. The diploid population and the bulked segregant analysis method were used to look for RAPD markers linked to non-nodulation mutation. At the same time the tetraploid segregating population was extended in order to determine the recombination frequencies in the region more precisely. Combining the two approaches resulted in identifying molecular markers in the close vicinity (1 cM) of the non-nodulation trait.

NODULE-STRUCTURE IN THE ROOTS OF GLEDITSIA SPP. A NON-NODULATING LEGUME GENUS

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The genus *Gleditsia* is phyllogenetically situated at the base of the family Leguminosae and it is supposed to be the origin of the nitrogen-fixing genera *Dimorphandra* and *Sclerolobium* (Polhill 1981,1994). The species of this genus do not nodulate, however the roots form some hypertrophic outgrowth. These structures were examined under light and electron microscopy. Bacteria were found inside structures very similar to infection threads in legumes. Immunoassay tests indicated the presence of nitrogenase in this material.

Plants were grown in pots containing sterilized soil and sand fertilized with nitrogen-free nutrient solution. The following treatments were studied: 1, control, 2, inoculation with a mixture of a nitrogen--fixing bacteria (NFB) isolated from acacias and other nitrogen fixing bacteria isolated previously from the roots of *Gleditsia triacanthos* (Dobereiner et al. 1995) and mycorrhizal fungi *Glomus clarum* and *Gigaspora margarita*; 3, inoculation with a mixture of NFB and 1 g of soil from a previous experiment containing native mycorrhizal fungi; 4, inoculation with a mixture of NFB and 1 g of soil from a previous experiment however from a different pot. The experimental design was randomized blocks with 5 replications. The plants were harvested 5 and 13 months after sowing. The plant dry weight and total N and P were evaluted. Root material was processed for light and electron microscope and the immunoassay against the nitrogenase was made according to Olivares et al. 1997.

Gleditsia triacanthos was shown to be a highly mycorrhizal dependent . After 5 and 13 months the plants inoculated with mycorrhizal fungi or soil grew more than the control plants The total N accumulated in the plant tissue was also increased in these treatment No differences in P accumulation were observed within the plants which received mycorrhizal fungi. No comparasion could be made with the control plants because insufficient plant material remained after samples were taken for examination under microscopy.The sections made in the roots segments and examined under light and electron microscopy showed structures very similar to infection threads ordinarily found in legume nodules inside plant cells. These "infection threads" crossed cell wall boundaries Apparently root plant cells do not became hypertrophic and show viable bacteria cells. These "infection threads" apparently are not surrounded by a membrane but the bacteria inside these structures are enclosed by a membrane. The control plants did not show these structures. Immunoassays against nitrogenase showed the presence of this enzyme. It is possible that these structures have preceeded actual nodules and these observations may contribute to undestand the origin of biological nitrogen fixation in the legume family.

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AUTOREGULATION OF NODULATION IN *VICIA SATIVA* SSP. *NIGRA.*

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Formation of, *Rhizobium*-induced symbiotic root nodules on leguminous plants such as *Vicia sativa* ssp. *nigra* (vetch) is subject to regulation by the plant. Once a certain number of root nodules is formed, the plant, in an unknown manner, stops the formation of new root nodules. This process is called autoregulation of root nodule formation (AUT) and represents a feed back response to the development of the first nodules. Since AUT most probably involves (plant)cell-to-cell signalling, a split root system was used in our study. In this system two lateral roots, root A and root B grown from the main root after cutting off its tip, are incubated each in a different tube.

With the vetch split root system we could demonstrate the following.

1) Signalling from a nodulating root A to root B results in a non-nodulating B root, and in acidification of B-root exudate. Acidification was not an absolute requirement for autoregulation, but can be one of more autoregulation factors.

2) Inducers of autoregulation and acidification signalling were: a) nodulating rhizobia, b) Nod Rlv (C18:4,Ac) and, c) NodRlv(C18:1,Ac) Nod factors. NodSm (C16:2,Ac,S) did not induce autoregulation and acidification.

3) Nod factors (C18:4) and (C18:1) are ,in this order, less efficient inducers of autoregulation than are nodulating rhizobia.

4) Inhibition of bacterial growth in B-root exudate is linked to acidification, as strong buffering prevented growth inhibition. Strong buffering only partly restored Ini {Induced *nod* gene inducing activity, (VanBrussel et al 1990)} which suggests that B-roots induced for AUT show impaired sensitivity to Nod factors.

5) The autoregulatory effect of non-mitogenic NodRlv(C18:1,Ac) implies that induction of root nodule primordia is not absolutely required for autoregulation, and is probably related to its inhibitory effect on polar auxin transport (Boot, Kees et al 1999) and its induction of the Thick and short roots (Tsr) phenotype (VanBrussel et al 1986).

6) Other bacterial factors inducing (partial) autoregulation of nodulation cannot be excluded.

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IDENTIFICATION OF SEQUENCES RELATED TO HEAT-STRESS GENES IN COWPEA GROWING UNDER BIOLOGICAL NITROGEN FIXATIONSCREENED BY cDNA-AFLP

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The reduction of plant growth and productivity caused by high temperature stress is especially important in semi-arid regions. This effect is even more severe when plants are growing under biological nitrogen fixation (BNF) (Piha & Munns 1987). High soil temperatures in tropical areas are a major problem for BNF activity of soybean, peanut and bean associate with rhizobia. It is responsible for strong deleterious effects on bacterial survival and infection, nodule development and as consequence reduction of N_2 fixation. The symbiosis between common bean and *Rhizobium* is considered to be especially high sensitive to temperature stress. Although several studies have investigated the microsymbiont tolerance, little is known about the plant (macrosymbiont). Furthermore, BNF is a complex process depending on the plant-bacteria-environment interaction.

To isolate plant genes related to high temperature stress responses under BNF conditions, we have used a new method to visualize differential gene expression based on AFLP so called cDNA-AFLP (Bachem et al.,1996). Cowpea plants inoculated with *Bradyrhizobium* sp, growing in a greenhouse at 28 °C for 15 days were submitted to high temperature stress (2h at 40 °C) in a growth chamber. The mRNA was separated from total nodule RNA of cowpea and cDNA was synthesized and used in the cDNA-AFLP experiments.

By using of the cDNA-AFLP strategy, we have identified in two different experiments, at least 60 transcript-derived fragments (TDFs), which were isolated from polyacrylamide sequencing gel, reamplified by standard PCR and used as probe in northern blot experiments in order to confirm their differential expression. The northern blot analyses have demonstrated that transcripts hybridizing with TDF F9.14 were present only in nodules submitted to high temperature stress. On the other hand when using TDF E3.4 as a probe, two different mRNAs were found: a ~1000 bp transcripts specifically expressed in nodules under heat stress and another with ~600 bp, which was constitutivelly present in leaves. Both fragments were cloned and sequenced. The analysis of these sequences showed no significant homology with known sequences in databank. Currently, we are performing RACE-PCR experiments to obtain full-length cDNA.

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NITROGEN FIXATION CHARACTERISTICS OF BRAZILIAN SOYBEAN CULTIVARS

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In Brazil, the soybean is cropped in almost 13 million hectares and plants rely mainly on biological nitrogen fixation (BNF) (about 170 kg of N ha^{-1}), with a small contribution of N from soil (about 15 to 30 kg of N ha^{-1}). However, although soybean breeding programs have been performed in soils with an established population of soybean bradyrhizobia, lately breeders have often neither inoculated the seeds nor evaluated the symbiotic performance, with more attention being paid to resistance to diseases and yield. This work aimed then to verify the variability among commercial soybean genotypes in relation to BNF parameters and to study the genetics of BNF of segregating populations obtained by the crossings of contrasting parental genotypes.

Evaluation of Soybean Cultivars for N_2 Fixation Capacity. In order to identify cultivars with higher BNF capacity, 152 North American and Brazilian soybean genotypes (maturity groups VI to VIII) were evaluated for nodulation parameters and BNF capacity. The cultivars were single inoculated (1 ml $seed^{-1}$ of an inoculum containing 10^9 cells ml^{-1}) with each one of the three *B. elkanii* strains established in most Brazilian soils: SEMIA 5019 (=29w), SEMIA 587 and SEMIA 566. Plants were grown under environmentally controlled and axenic conditions (Leonard jars under greenhouse conditions with plants receiving N-free nutrient solution). None of the cultivars tested restricted completely the nodulation, although up to 4 fold differences in nodule number and mass and 2.5 fold differences in total N accumulated in plants were found. Effects of cultivars, strains and the interaction among cultivars and strains were verified for the parameters of nodule number and dry weight, shoot dry weight and total N accumulated in tissues. Many recently released cultivars had a decreased N_2 fixation capacity in relation to the parental genotypes. The results obtained under axenic conditions were confirmed in another experiment conducted in pots containing 4 kg of soil with an established population of the three *B. elkanii* strains (29w : SEMIA 566 : SEMIA 587, 22% : 36% : 34%, respectively).

Study of genetics of biological nitrogen fixation. To investigate the inheritance of quantitative traits of BNF, complete dialel crosses (direct and reciprocal) of three parental cultivars, Bossier (natural mutation in cv. Lee), J-200 (IAC-2 X Viçoja), both showing a high BNF capacity and Embrapa 133 (FT-Abyara X BR 83-147), with medium BNF capacity, were performed. Segregating generations F_2 and F_3 were obtained by the single seed descent (SSD) method. In the crossing of J-200 and Embrapa 133, effects of dominance [h] were verified for nodule number and dry weight, therefore showing genetic variability among the parental genotypes. In the crossing of Bossier (good N_2 fixer) and Embrapa 133, additive [d], dominance [h] and epistasy [i] effects were detected just for the nodule number parameter, therefore allowing a search for molecular markers linked to the nodulation genes.

In Brazil, the economical viability of the soybean crop is based on an efficient supply of N by the biological process. Consequently, the parameters of nodulation and N_2 fixation capacity should always be consider as a priority in the breeding programs, otherwise the new cultivars can have a decreased symbiotic capacity.

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CLONING OF GSI-LIKE GENES OF *MEDICAGO TRUNCATULA*: SUPPORT FOR THE PARALOGOUS EVOLUTION OF GSI AND GSII GENES

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1. Introduction

In our studies of symbiotic gene expression in the model legume, *Medicago truncatula,* we have identified a gene, *MtN6,* which is expressed in roots during the events preceding infection by *Sinorhizobium meliloti* (Mathis et al. 1999a). This gene shows homology to the N-terminal domain region of *fluG,* which is a key regulator of sporulation in the fungus *Emericella nidulans* (Lee, Adams 1994). As activity of FluG depends on its C-terminal domain, we decided to look *for M. truncatula* clones related to this domain, whose encoded protein may interact with MtN6.

The C-terminal domain of FluG shows homologies to the GSI group of glutamine synthetases (GS). Two types of GS have been described, the typical prokaryotic enzyme, GSI, and the typical eukaryotic enzyme GSII. These two enzymes show only about 25% amino acid identity. From a phylogenetic analysis, it has been pointed out that GSI and GSII genes probably diverged before the evolution of eukaryotes and therefore both types of genes should exist in both types of organisms (Kumada et al. 1993). Although GSII genes occur in a growing list of bacteria, *fluG* is the only eukaryotic gene published to date showing homology specifically to GSI. Therefore, whether these two types of genes are really paralogous has not been resolved.

2. Results

Using a GSI-related clone identified from the *Arabidopsis* EST banks, we have isolated a number of clones related to GSI from root cDNA libraries of *M. truncatula.* One of these clones correlated to a full-length mRNA of about 1.7 kb and was completely sequenced. It encoded a protein of 454 amino acids showing only about 24% identity to GSII proteins but about 40% identity to prokaryotic GSI sequences. Phylogenetic analysis showed that this protein and FluG form a cluster as the most divergent GSI sequences and are clearly distinct from all GSII sequences (Mathis et al. 1999b). Screening of EST and other databases revealed the presence of GSI-like genes in both monocotyledonous and dicotyledonous plants but not in other eukaryotes including yeast and *C elegans.*

3. Conclusion

GSI-like genes appear to be widespread in higher plants. This discovery strongly supports the paralogous evolution of GSI and GSII genes and suggests that the lack of one or other type of gene in most eukaryotic or prokaryotic species so far studied, is probably due to subsequent loss during evolution, perhaps due to functional redundancy.

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CATION TRANSPORT IN SOYBEAN ROOT NODULES

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Nitrogen-fixing bacteroids of leguminous plants are housed within the peribacteroid membrane (PBM) of plant root nodules. Nutrient transport from the plant cell cytosol, across the PBM, is essential for bacteroid metabolism and nitrogen fixation. The major metabolic exchange across the PBM is reduced carbon (malate) for fixed nitrogen. However, the bacteroids are dependent on the plant for many micronutrients also and these must also be transported across the PBM. Iron is of particular importance as it is an integral component of several key symbiotic proteins, including nitrogenase, leghemoglobin and cytochromes. Uptake studies using radioactive-iron with isolated symbiosomes and bacteroids from soybean nodules have identified two transport systems for iron on the PBM. Iron can be transported either in the form of ferric-citrate (LeVier et al. 1995; Moreau et al. 1995) or as ferrous iron (Moreau et al. 1998). Citrate, for chelating iron, is present in significantly high amounts in the nodule cytosol and is the transport form of iron in soybean xylem. However, ferrous iron has been found to cross the PBM more efficiently than ferric-citrate (Moreau et al. 1998). Ferrous iron transport across the PBM is in agreement with the reducing conditions found within nodules which would stabilise ferrous iron. Isolated bacteroids are able to take up ferrous iron more rapidly than the ferric form.

We have isolated from a soybean nodule library two cDNAs which show homology to members of gene families encoding membrane transporter proteins in other organisms. One of these cDNAs encodes a protein which is similar to both *IRT* and *ZIP* gene products. Respectively, IRT and ZIP proteins from Arabidopsis have been found to preferentially transport Fe^{2+} (Eide et al. 1996) and Zn^{2+} (Grotz et al. 1998). Immunoblot analysis, using an antibody raised against AT IRT1, shows the presence of an IRT-like protein on the PBM. Our other candidate iron transporter is similar to members of the *Nramp* gene family (Cellier et al. 1996) which encode proteins able to transport a broad range of divalent cations including iron (Gunshin et al. 1997). Both the soybean *IRT/ZIP* and *Nramp* genes have nodule-enhanced expression, suggesting that they might be located on the PBM. To determine the cation specificity of the soybean IRT/ZIP and Nramp we will perform functional analysis in yeast mutants which are deficient in either iron or zinc transport.

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THE BROAD BEAN NODULIN VFENOD18: EXPRESSION ANALYSIS AND IMMUNOLOGICAL LOCALIZATION IN *VICIA FABA* L. NODULES

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The symbiotic interaction of broad bean (*Vicia faba* L.) with the soil bacterium *Rhizobium leguminosarum bv. viciae* leads to the formation of nitrogen fixing root nodules, where nitrogen fixation is carried out by the microsymbiont. The plant expresses specific genes, designated nodulins, in the root nodules. By cross-hybridization and sequencing of a nodule specific cDNA library, we identified transcript sequences representing 44 different genes active in broad bean root nodules (Perlick, Pühler, 1993).

Two transcript sequences isolated from this nodule cDNA library represented the nodulin VfENOD18. Northern blots revealed that VfENOD18 is exclusively expressed in root nodules. Using tissue-print hybridizations we localized VfENOD18 transcripts in the nitrogen fixing zone of nodules. Time course experiments demonstrated that the onset of VfENOD18 expression was at least one day before the start of leghemoglobin gene expression, which is correlated with the start of nitrogen fixation. Therefore, VfENOD18 represents an early nodulin.

Ten genomic clones carrying VfENOD18 sequences were identified. Sequence analyses of the genomic clones revealed the existence of a VfENOD18 gene family, consisting of two classes of isogenes. The sequence of a 1996 bp potential promoter of the class II isogene showed no similarities with promoter elements from other nodulin promoters. To study the activity of this promoter, Gus-fusions were tested in transgenic roots and nodules of *Vicia hirsuta.* Histochemical analysis revealed that VfENOD18 promoter elements located on a -964 / -14 subfragment are sufficient for the exclusive expression of the reporter gene in the nitrogen fixing zone. VfENOD18 transcripts code for a nodulin of 18.5 kD. Database searches revealed homologies to plant ESTs of yet unknown biological function. The VfENOD18 amino acid sequence also displayed significant homologies to a variaty of bacterial proteins (e.g. of *Methanococcus jannaschii* and of *Bacillus subtilis*) including members of a protein family referred to as MJ0577 superfamily (Zarembinski et al., 1998). The recently described crystal structure of the *M. jannaschii* protein MJ0577 reveals that this protein binds ATP, suggesting that MJ0577 is an ATPase or an ATP-mediated molecular switch (Zarembinski et al., 1998). The majority of the amino acid residues involved in the ATP binding and the dimerization of MJ0577 are conserved in the homologues of VfENOD18.

Using antibodies generated against recombinant VfENOD18 protein we demonstrated the nodule-specifity of VfENOD18. Furthermore, VfENOD18 antibodies recognized homologous proteins in extracts from indeterminate, but not in extracts from determinate legume nodules. To learn more about the function of VfENOD18 in broad bean nodules we carried out localization experiments. VfENOD18 protein was localized by light microscopy of longitudinal sections of broad bean nodules following immunogold labeling and silver enhancement. Signals were found in the nitrogen fixing zone, consistent with the results obtained for VfENOD18 transcripts and the activity of the VfENOD18 promotor. Electron microscopy of *Vicia faba* nodule tissue treated with VfENOD18 antibodies revealed that VfENOD18 proteins are localized in the cytoplasm of infected cells.

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REGULATION OF UREIDE SYNTHESIS IN COWPEA NODULES

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cDNAs encoding all nine enzymes of the *de novo* purine pathway (*pur* genes) have been cloned from cowpea nodules. Polyclonal antibodies to recombinant proteins of GART and AIRS and to native urate oxidase have been used to determine aspects of their regulation. Transient exposure of nodulated root systems to atmospheres devoid of N_2 (80% Ar:20% O_2) causes the activity of these enzymes to decline. In the case of urate oxidase and GART this was due to post-translational modifications but for AIRS transcriptional control was indicated. Expression of *pur* genes when nodules develop in $Ar:O_2$ was also investigated. Although mRNA for the *pur* genes was detected in these nodules there was no enhancement of transcription after nitrogenase became active. This contrasts with results for air-grown plants where expression is increased after N_2 fixation begins. Our results suggest a tight link between the products of N_2 fixation and regulation of the purine biosynthesis pathway in nodules. However the mechanisms for regulation of different genes and enzymes differ and there is control at both the transcriptional and post-transcriptional levels.

RHIZOBITOXINE PRODUCTION OF *BRADYRHIZOBIUM ELKANII* ENHANCES SIRATRO NODULE FORMATION

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Rhizobitoxine, a *Bradyrhizobium elkanii*-producing ethylene synthesis inhibitor, has been thought as a potent factor to enhance nodule formation, since ethylene blocks nodule formation in some legumes. Ethylene evolution rate in siratro roots inoculated with *B. elkanii* USDA94 was 0.62 pmole/plant/hour 3 days after inoculation (DAI), while uninoculated control roots was 4.91 pmole/plant/hour. This result indicates that inoculation with a rhizobitoxine producer reduces ethylene evolution in the inoculated roots. Addition of 1-aminocyclopropyl-l-carboxylic acid, an ethylene precursor, increased ethylene evolution rate, and reduced nodule formation in siratro (*Macroptilium atropurpureum*) roots with *B. elkanii* USDA94. These results suggest that ethylene affects siratro nodule formation. Then, we examined whether a bacterial production of rhizobitoxine enhances nodule formation, using *Bradyrhizobium-Macroptilium atropurpureum* system. A rhizobitoxine production deficient mutant RTS2 was constructed from USDA94 by homologous recombination with a rhizobitoxine production related gene *rtxA* (Ruan et al 1993). Rhizobitoxine production was determined according to Yasuta et al. (Yasuta et al 1999). Rhizobitoxine concentration of the constructed mutant RTS2 in the culture supernatant was at an undetectable level (< 0.01 □M), while that of parent strain USDA94 was 17.5 □M. Ethylene evolution in siratro roots 23 DAI with the mutant RTS2 was 6.63 pmole/plant/hour, while that of the parent was at an undetectable low level. In a comparative time course study of nodule formation with the parent USDA94 and the mutant RTS2 a significant difference was observed only 12 days after inoculation (DAI). It is possible that rhizobitoxine is accumulated during growth of the host plant (Minamisawa, Kume 1987) and that the resultant high concentrations of rhizobitoxine, due to inhibition of ethylene synthesis, increases the development of nodules. In multistrain environments of *Bradyrhizobium,* nodule occupancy of *B. elkanii* was significantly higher than that of *B. japonicum* in siratro (Minamisawa et al, 1997). We examined whether a bacterial production of rhizobitoxine increases competitive nodule formation ability. Co-inoculation studies using the rhizobitoxine production deficient mutant RTS2 indicate that lack of the ability of rhizobitoxine production in *B. elkanii* reduced its nodule occupancy in competition against the parent *B. elkanii* USDA94 and *B. japonicum* USDA110. These results suggest that rhizobitoxine produced by *B. elkanii* increase the ability of competitive nodulation under intra-species and inter-species competitions in *Bradyrhizobium.*

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A NEW CLASS OF PLANT DYNAMIN HOMOLOG IS STRONGLY EXPRESSED IN NODULES OF *ASTRAGALUS SINICUS* CV. JAPAN

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Astragalus sinicus is one of legumes widely cultivated in Japan and China. We identified nodule-specific genes using differential display, among them is a plant dynamin homologue *AsDH1* (*Astragalus sinicus* dynamin homologue 1). We cloned the full length cDNA of *AsDH1* which is homologous to known plant dynamins, phragmoplastins from soybean and ADLs from *Arabidopsis.* As in other plant dynamins, there are three putative GTP-binding domains. At the C-terminus of deduced amino acid sequence, there is a PH domain, recognized only in ADL3. *AsDH1* is highly expressed in developed nodules, but also in other tissues in a small amount.

SECTION VII

METABOLISM IN SYMBIOSOMES

SYMBIOSOME METABOLISM IN LEGUME NODULES

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1. Symbiosome Structure and Biogenesis

The term symbiosome was invoked by Roth et al (1988) to describe the organelle-like structure formed upon endocytosis of a microsymbiont by a host cell and was defined as "a membrane-bound compartment containing one or more symbionts and certain metabolic components and located in the cytoplasm of eukaryote cells". The structure of a symbiosome from soybean is shown in Fig. 1: it consists of a host derived membrane (the symbiosome membrane) which envelopes a microsymbiont and creates an internal space called the symbiosome space. In legumes, the symbiosome membrane is generally referred to as the peribacteroid membrane (PBM).

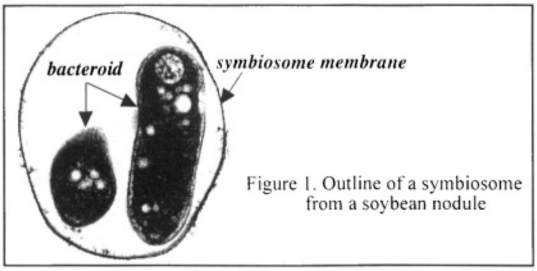

Figure 1. Outline of a symbiosome from a soybean nodule

Rhizobia penetrate the cortex of roots via an infection thread and enter the inner cortical cells via endocytosis of the infection thread membrane following hydrolysis of the infection thread wall (Fig. 2; see Whitehead and Day 1997 for a review). The newly formed symbiosomes proliferate through the cell as bacteria and PBM divide in concert. Differentiation of the rhizobia into nitrogen-fixing bacteroids follows. In determinant nodules such as soybeans, the PBM of adjacent symbiosomes appears to fuse, forming multiple bacteroid symbiosomes (Fig.2; Fedorova et al 1999). Mature infected cells of legume nodules contain many thousands of multiple bacteroid symbiosomes.

2. Nutrient Exchange Across Symbiosome Membranes

The major nutrient exchange between bacteroid and plant is fixed nitrogen from the bacteroid for a source of reduced carbon from the plant. There appears to be a consensus that the major form of carbon supplied to the bacteroid is malate (or other dicarboxylates). However, the form in which nitrogen leaves the symbiosome remains controversial.

Historically, ammonia has been generally considered to be the major product of nitrogen fixation to leave the bacteroid, and the identification of an ammonium channel on the PBM appeared to confirm this (Tyerman et al., 1995; Mouritzen et al., 1997).

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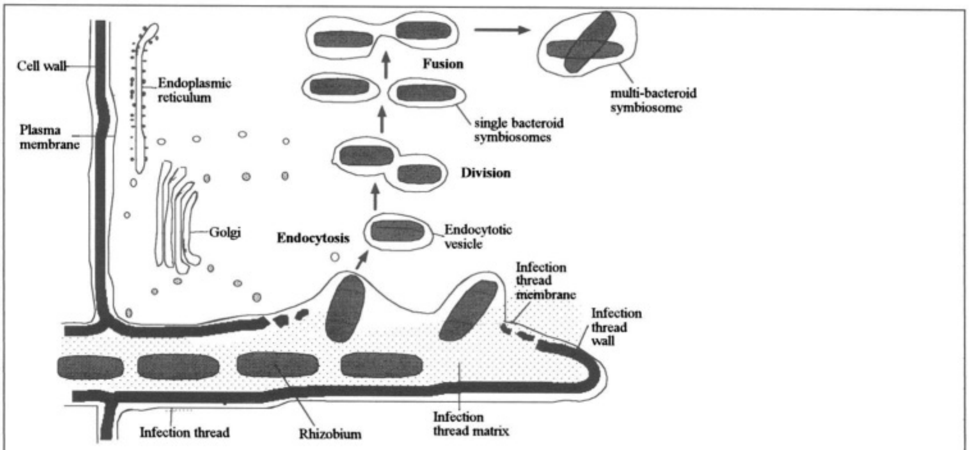
Cell wall
Endoplasmic reticulum
Plasma membrane
Fusion
multi-bacteroid symbiosome
single bacteroid symbiosomes
Division
Golgi
Endocytosis
Endocytotic vesicle
Infection thread membrane
Infection thread wall
Infection thread
Rhizobium
Infection thread matrix

Figure 2. Schematic outline of symbiosome formation in soybean nodules. Modified from Whitehead and Day (1997).

However, amino acids have been often considered as alternatives to ammonia (eg, Kahn et al., 1985) and efflux of amino acids, most noticeably alanine and aspartate, has been reported from isolated bacteroids and symbiosomes (Kouchi et al 1991, Rosendahl et al 1992, Appels and Haaker 1991). More recently, an **aspartate/H$^+$** carrier has been identified on the PBM of pea nodules (Rudbeck et al 1999) and labeling of alanine from $^{15}N_2$ has been reported in isolated soybean bacteroids (Waters et al., 1998). Our own investigations in soybean have shown that some alanine (and possibly other amino acids) is formed when conditions which favour ammonia accumulation and restrict NADH oxidation by the respiratory chain, are imposed in vitro (see Li et al, this volume). In vivo, the product of nitrogen fixation that is provided to the plant may be determined by the demand in the plant for ammonia and the rate of supply of malate and oxygen.

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ASSIMILATION OF FIXED NITROGEN STUDIED BY MEMBRANE VESICLE TRANSPORT AND *IN VIVO* ^{15}N-NMR SPECTROSCOPY IN PEA

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1. Introduction

In legume nodules the majority of the dinitrogen reduced by the bacteroids is transferred to the plant as the enzymes of the ammonium-assimilation pathway, which operates in free-living bacteria, are repressed in the symbiotic bacteroids (Taté et al. 1998). The nitrogenase-generated ammonium may be transferred by diffusion of ammonia across the bacteroid membrane and subsequently transported as ammonium across the symbiosome membrane via an ion channel (Tyerman et al. 1995; Mouritzen, Rosendahl 1997). A nutrient exchange model in which nitrogenase-generated ammonium is assimilated into a carbon skeleton before it is exported from the symbiosome was proposed by Kahn et al. (1985). Dicarboxylic acids from the plant supply the bacteroids with the carbon needed to fuel nitrogen fixation (Udvardi, Day 1997) but a significant proportion of the dicarboxylic acids supplied *in vitro* to nitrogen-fixing bacteroids is converted to amino acids within the symbiosome (Salminen, Streeter 1990). Kinetic analysis of the plant aspartate aminotransferases indicated that aspartate synthesis in the plant was not sufficient to provide enough precursors for the asparagine exported from amide transporting nodules (Haaker et al. 1995). It was proposed that malate taken up by the symbiosomes may be aminated in the symbiosome to form aspartate, which is then exported from the symbiosome as an additional source of precursor for asparagine synthesis (Haaker et al. 1995). In accordance with this amino acids, primarily aspartate and alanine, have been shown to be exported from nitrogen-fixing bacteroids and symbiosomes, when they are provided with dicarboxylic acids as a carbon source in *in vitro* studies (Appels, Haaker 1991; Rosendahl et al. 1992; Waters et al. 1998). Here we review the possible transfer of fixed nitrogen as amino acids from the bacteroids to the plant, and present for the first time a non-invasive reflection of the assimilation of fixed nitrogen in nodules obtained by *in vivo* ^{15}N-NMR spectroscopy.

2. An amino acid transporter in the symbiosome membrane identified by membrane vesicle transport

Transport of compounds across the symbiosome membrane is driven by a membrane potential ($\Delta\psi$) and a pH gradient (ΔpH), which is generated by the proton pumping ATPase associated with the symbiosome membrane (Christiansen et al. 1995). The end result is a $\Delta\psi$, of around –100 mV (Tyerman et al. 1995) and a ΔpH, which is estimated to be in the range of 1 – 1.6 pH units (Udvardi et al. 1991). Thus the proton pumping ATPase of the symbiosome membrane generates a positive charge and an acidic environment on the bacteroid side of the symbiosome membrane compared with the plant cytosol (Figure 1). The majority of studies on transport mechanisms in the symbiosome membrane have been designed to study transport directed into symbiosomes. Studies

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of transport mechanisms directed from the bacteroid side to the plant cytosol are methodologically more complicated. However, we have established a method whereby sealed bacteroid side-out symbiosome membrane vesicles may be purified from symbiosomes and subsequently used for studies of transport mechanisms directed from the bacteroid to the plant (Mouritzen, Rosendahl 1997). A controlled $\Delta\psi$ is imposed on the membrane vesicles using a K^+/valinomycin clamp, and a ΔpH across the membrane is obtained by resuspending membrane vesicles loaded with a buffer of a defined pH in a transport buffer of varying pH. In this way energised bacteroid side-out symbiosome membrane vesicles are constructed with an artificially imposed $\Delta\psi$, intravesicular negative, and a ΔpH, intravesicular alkaline, to imitate the *in nodule* proton electrochemical gradient across the symbiosome membrane.

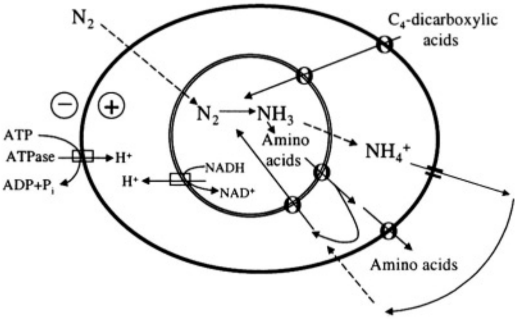

Figure 1. Nitrogen fixation and transfer in the symbiosome.
Double-line circle, bacteroid membrane; single-line circle, symbiosome membrane; dashed-line arrows, diffusion; full-line arrows, transport mechanism; ***O****, carrier-mediated transport; =, channel mediated transport.*

Energised bacteroid side-out symbiosome membrane vesicles accumulated aspartate from the extravesicular medium in a time-dependent manner and against a concentration gradient (Rudbeck et al. 1999). The transport of aspartate into the vesicles was electrogenic and both components of the electrochemical potential, $\Delta\Psi$ and ΔpH, contributed to the transport. A saturable component of the transport was observed at extravesicular aspartate concentrations below 1mM and a Hanes plot in this range revealed a K_m of 220 mmol m^{-3}. Transport of aspartate was inhibited by protein modifying reagents. Taken together the results correspond with the presence of a proton/aspartate symport that may transport aspartate out of symbiosomes in pea nodules. The identified transporter has a K_m for aspartate in the same range as the bacteroid general amino acid permease (Aap), which is substantially lower than that of the bacteroid dicarboxylic acid transport (Dct) system, the K_m for aspartate transport of free-living bacteria, and that of the plasma membrane (Table 1). The amino acid specificity of the identified transporter has not been determined yet. Therefore it remains to be established if the symbiosome membrane possesses a facilitator for transport of alanine, which is another likely candidate for transfer of fixed nitrogen as an amino acid out of the symbiosome.

Table 1. Aspartate transporter kinetics

Membrane	(Transp.)	K_m (μM)	V_{max} (nmol/min/mg)	References
Bacteria				
S. meliloti	(Dct)	10,000		*Watson et al. 1993*
S. meliloti	(Aap)	1,500		*Watson et al. 1993*
Bacteroid				
S. meliloti	(Dct)	10,400	75.8	*McRae et al. 1989*
R. legumino.	(Aap)	28	0.7	*Herrada et al. 1989*
B. japonicum	(Aap)	105	2.9	*Salminen, Streeter1991*
B. japonicum	(Aap)	28	5.1	*Whitehead et al. 1998*
Symbiosome				
Pea-*R. legumino.*		220	4.8	*Rudbeck et al. 1999*
Plasma				
Ricinus communis		1,616	58.8	*Westen et al. 1995*

3. *In vivo* ^{15}N-NMR on nodules

The significance of symbiosome amino acid synthesis and export in the overall assimilation of fixed nitrogen in legume nodules remains to be determined. The presence of an amino acid transporter and an ammonium transporter in the symbiosome membrane reflects that fixed nitrogen may leave the symbiosome in either form. However, even with the knowledge on the capacity of the individual transporters in mind, their *in nodule* significance can only be estimated, since information on the *in nodule* micro-environmental conditions of the symbiosomes is based on estimates. A true reflection of the assimilation of fixed nitrogen in nodules requires non-invasive and non-destructive measurements of the fate of the nitrogen fixed. *In vivo* ^{15}N-NMR spectroscopy may provide this possibility. Ideally, *in vivo* ^{15}N-NMR spectroscopy can provide real-time information on identity, levels, localisation, and environment of intracellular free nitrogen metabolites during a time course, when the individual metabolites accumulate in pools that are large enough for detection.

We have constructed a gas tight perfusion system, which is connected to an NMR tube. In this system root nodules can be maintained metabolically active in the NMR tube for at least 12 hours. The physiological condition of the nodules was monitored by ^{31}P-NMR spectroscopy, which demonstrates a constant and high ATP/ADP ratio (A.M. Scharff, P.E. Hansen and L. Rosendahl, unpublished). $^{15}N_2$ was introduced into the perfusion system and nodule assimilation of the nitrogenase-fixed nitrogen into amino acids was followed in a time course study. The first ^{15}N labelled amino acid to exceed the detection limit was glutamate, which appeared first in the NMR spectrum. Subsequently ^{15}N was detected in the amide of asparagine (A.M. Scharff, unpublished). A limitation of NMR spectroscopy is a rather high detection limit and a decent pool size is therefore required for a metabolite to be detected. However, an obvious advantage of the method is the fact that information is obtained on the exact position of the ^{15}N in a given metabolite. Extracts from root nodules, which had been incubated with $^{15}N_2$, were analysed by GC-MS in order to identify the overall ^{15}N enrichment in amino acids. The GC-MS data demonstrated ^{15}N enrichment in glutamine, γ-aminobutyrate, glutamate, alanine, asparagine, and aspartate, in descending atom% enrichment. The inability to detect all of the ^{15}N-enriched amino acids in the ^{15}N-NMR spectrum may be ascribed to small pool sizes of some of the ^{15}N-enriched amino acids. Further optimisation of the *in vivo* ^{15}N-NMR procedure is required in order to get a complete reflection of the *in nodule* assimilation pathway for fixed nitrogen.

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ALANINE EXCRETION FROM *Bradyrhizobium japonicum* BACTEROIDS

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1. Introduction

The ammonia diffusion hypothesis has been the prevalent model surmising the initial steps of leguminous nodule nitrogen transfer (Day review 1997). The product of nitrogenase exists in two forms within the bacteroid, ammonium [NH_4^+] and ammonia [NH_3], which are readily interconvertible. The pKa of the ammonium/ammonia equilibrium is pH 9.26. At an intracellular pH of 8.26 only 10% of the total exists as ammonia. The ammonium ion can not diffuse across membranes, but ammonia is believed to diffuse freely out of the bacteroid. At the presumed intracellular pH within the bacteroid there would be a considerable buildup of ammonium to permit the small amount of ammonia to diffuse out of the bacteroid. Diffusion also requires a low extracellular concentration of ammonium/ammonia. It has not been unequivocally demonstrated that these condition exist within nodules. Once outside the bacteroid, but within the symbiosome space, the ammonia may either diffuse or be transported across the symbiosome membrane (Tyerman *et al,* 1995) where it is assimilated by the host plant into glutamate by the concerted action of glutamine synthetase and glutamate synthase.

A compounding factor in many studies on bacteroid metabolism and functioning has been the degree to which these preparations are contaminated with plant enzymes and metabolites. Bacteroid preparations deemed microscopically pure have been found to be contaminated with plant enzymes (Karr *et al,* 1988, Karr, Emerich, 1990). The presence of plant enzymes and metabolites can significantly alter results concerning the metabolic products released from bacteroids. For example, the presence of plant aminotransferase and amino acid dehydrogenases could produce ammonium from a nitrogen-carrying molecule transported out of the bacteroid.

In 1985, Kahn *et al.,* proposed a nutrient exchange model in which the nitrogenase-generated ammonium was first assimilated into a carbon skeleton and then carried across the bacteroid membrane. Glutamate and aspartate were suggested as candidates to participate in membrane transversing shuttles such as the malate-aspartate shuttle. Recently, Waters *et al.,* (1998) have presented evidence that alanine, and not ammonium, is the primary nitrogen compound transported out of the bacteroid. Here we will present some of the experimental results supporting alanine as the primary nitrogen-containing molecule exported from soybean nodule bacteroids.

2. Procedures.

Soybeans (*Glycine max* L. cv. Williams 82) were inoculated with either *B. japonicum* strain 110 or 2143 and grown as previously described (Karr *et al,* 1988, Karr, Emerich, 1990). Bacteroids were isolated anaerobically under argon by the sucrose density gradient procedure of Waters *et al.* (1985). Procedures for alanine excretion, ammonium excretion, and ^{15}N analysis can be found in Waters *et al.,* (1998). Amino acid analysis was performed on Beckman 6300 amino acid analyzers. Details of the purification and enzymatic characterization of *B. japonicum* bacteroid alanine dehydrogenase can be found in Smith and Emerich (1990a,b).

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3. Results

Sucrose density gradient purified bacteroids were incubated under nitrogen-fixing conditions and the exudates were collected as a function of time and each sample was analyzed by amino acid analysis. Alanine was the only compound that increased reproducibly and linearly with time in N_2 fixation assays (Figure 1). The excretion of alanine persisted in the presence of malate for up to 80 minutes. The excretion of alanine was markedly reduced in the absence of malate, but it remained the only nitrogen-containing compound that increased with time.

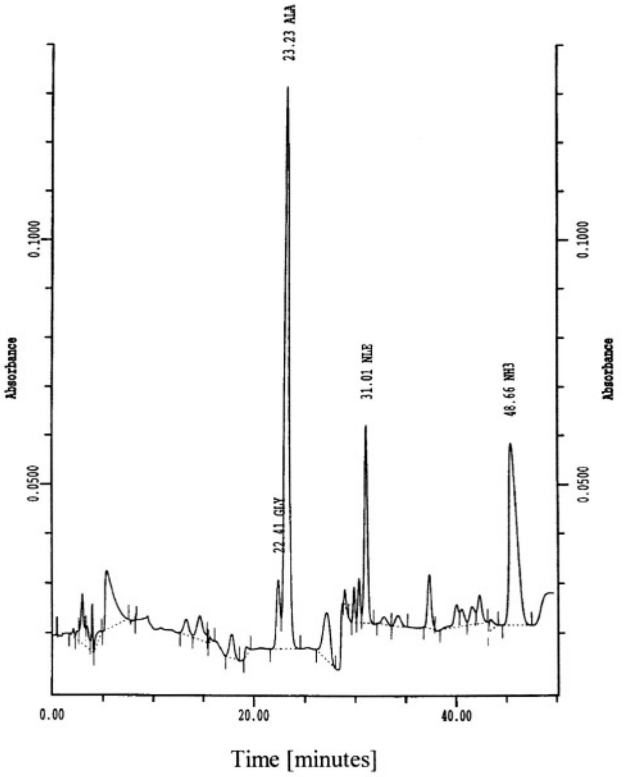

Figure 1. Amino acid analysis of exudate from sucrose density purified *B. japonicum* bacteroids. Alanine elutes the column at 23.23 minutes and ammonium at 48.66 minutes. Norleucine (NLE) is included as an internal standard and elutes from the column at 31.01 minutes. The X-axis is time in minutes and the Y-axis is absorbance after treatment with ninhydrin.

Ammonium was present in all assays as an impurity in the reagents and through absorption of ammonia from the atmosphere. Ammonium did not accumulate during the N_2 reduction assays of sucrose density gradient purified bacteroids, but more commonly was observed to slowly decline over time.

Since alanine was the only nitrogen-containing compound excreted from the bacteroids, it was suspected to be the carrier of ammonium generated by nitrogenase. To determine whether the alanine, indeed, was the carrier of reduced nitrogen out of the bacteroid, sucrose density gradient purified bacteroids were incubated under 99.4 atom% excess $^{15}N_2$, and the compounds found in the assay medium were analyzed by gas chromatography-mass spectrometry. The only compound that contained significant amounts of ^{15}N was alanine. The ^{15}N content of alanine was greater than 98 atom% excess. The ^{15}N content of the ammonium obtained from theses assay solutions typically contained less than 0.06 atom% excess.

The alanine that is excreted from *B. japonicum* bacteorids was formed by an alanine dehydrogenase that has an ordered Ter-Bi Theorell-Chance mechanism with rapid-equilibrium binding of ammonium in the second position (Smith, Emerich, 1993a,b). The ammonium does not simply diffuse to the enzyme active site and react chemically with the enzyme-substrate complex, but rather appears to bind directly to the enzyme. This suggests why the apparent K_m for ammonium of the *B. japonicum* enzyme was substantially lower compared to the alanine dehydrogenases of other microorganisms. The enzyme preferentially binds ammonium rather than ammonia, which permits *B. japonicum* alanine dehydrogenase to use the form that would be most abundant at the physiological pH within the bacteroid. Ammonium binds and dissociates very rapidly, but the following catalytic step is theoretically infinite, which permits extremely rapid assimilation of ammonium. In a Theorell-Chance mechanism, the rate of catalysis and the release of the first product, in this case alanine, are theoretically infinite (Smith, Emerich, 1993b). Therefore, a greater percentage of the ammonium that binds to the active site will be converted to products before it has time to dissociate. This demonstrated that *B. japonicum* alanine dehydrogenase was an extremely powerful ammonium assimilatory enzyme, unlike most alanine dehydrogenases, which have a catabolic role (Smith, Emerich, 1993a). In agreement with the catalytic mechanism, the amount of ^{15}N in ammonium within the bacteroid under conditions in which alanine excretion occurs was at the limit of detection suggesting little free ammonium exists within the microsymbiont. Also, when exogenous ammonium was added to bacteroids under N_2-fixing conditions, alanine excretion increased implying that the formation of ammonium from nitrogenase was limiting and not the synthesis of alanine.

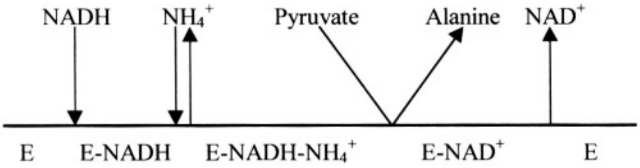

Figure 2. **Kinetic mechanism of *B. japonicum* alanine dehydrogenase. The vertical arrows represent the addition of substrates in Michaelis-Menten fashion. Ammonium binds to the enzyme in a rapid equilibrium fashion directly to the enzyme. The slanted arrows represent Theorell-Chance addition of pyruvate and release of alanine. The various forms of the enzyme (E) are indicated below the horizontal line. A Theorell-Chance reaction mechanism does not have a central complex.**

4. Discussion

The results presented here and by Waters *et al.* (1998) clearly demonstrate first, that alanine was the primary compound exported from bacteroids carrying nitrogenase-generated ammonium, and second, that a transport mechanism rather than diffusion functions at this critical step of nitrogen transfer from the bacteroid to the plant host. The incorporation of $^{15}N_2$ into alanine and not ammonium is compelling evidence for carrier mediated transport of fixed nitrogen from the bacteroid. Ammonium was not found to be released under the conditions used. The primary reason for the absence of ammonium is due to the fact that our isolation and assay conditions retard the primary source of ammonium released from bacteroid: protein degradation. In addition to demonstrating that alanine is the primary nitrogen compound transported from bacteroids, we have been able to demonstrate that ammonium release is the result of protein degradation and the release of the amino acid amide group as ammonium (data not presented).

Alanine is synthesized by an alanine dehydrogenase, which possesses a unique Theorell-Chance mechanism that strongly promotes the synthesis of this amino acid. The powerful synthetic activity of *B. japonicum* alanine dehydrogenase was evident in the ability of the bacteroid to assimilate exogenous ammonium into alanine (Waters *et al.*, 1998). This implies that alanine excretion was limited by the availability of ammonium produced by nitrogenase.
The results presented here and by Waters *et al.*, (1998) mandate new investigations of nodule nitrogen metabolism, in particular, the metabolic fate of alanine after it leaves the bacteroid. Alanine is most likely only a transport species as glutamate is the central compound of nitrogen metabolism in most organisms. The use of alanine as a transport species would functionally and spatially separate and distinguish bacteroid nitrogen metabolism and transport from bacteroid, symbiosome and plant host cellular nitrogen metabolism, thereby facilitating the rapid movement of fixed nitrogen from the bacteroid to the plant.

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CARBON METABOLISM AND SYMBIOTIC NEEDS OF ROOT NODULE BACTERIA

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1. Introduction

During the symbiotic association between members of the *Rhizobiaceae* and their plant hosts a series of complex reactions occur. Chief among these is the transfer of reduced carbon sources from the plant to the bacteroid where they are oxidized to produce the energy required for nitrogen fixation. The reduced nitrogen is donated to the plant host resulting in enhanced growth under nitrogen limiting conditions. This complex relationship requires that both bacteria and plant maintain compatible systems for the import, export and utilization of these compounds.

2. Carbon Sources Available from the Plant Host

The dominant C-compounds, which are available for metabolism by bacteroids in the root nodules, are the C_4 dicarboxylic acids such as succinate and malate (Vance and Heichel, 1991). Photosynthate, namely sucrose, is translocated to the nodule and metabolized to organic acids such as phosphoenolpyruvate (PEP) and this is believed to be converted to malate by the high levels of PEP carboxylase and malate dehydrogenase present in the plant tissue. Several $^{14}CO_2$ labeling studies with nodules have shown that malate is the dominant carbon source produced (Vance et al., 1983; Kouchi and Nakaji, 1985; Snapp, Vance, 1986; Rosendahl et al., 1990).

3. Importation Systems of Peribacteroid Membrane and Bacteroids

To be utilized by the metabolic machinery of the bacteroid, the carbon sources must traverse both the host-derived peribacteroid membrane and the bacteroid membrane. The peribacteroid membrane is relatively impermeable to sugars but does contain a dicarboxylate transporter capable of rapidly transferring C_4-dicarboxylic acids to the bacteroid (Streeter, 1987, Day et al., 1989). Furthermore, bacteroids themselves are capable of taking up C_4-dicarboxylates at rates significantly higher than that for sugars such as glucose (Salminen, Streeter, 1987; Udvardi et al., 1988). Mutants of rhizobia that are deficient for C_4-dicarboxylate transport are uniformly unable to fix atmospheric nitrogen in association with their plant hosts (e.g., Ronson et al., 1981; Finan et al., 1983). This evidence suggests that C_4-dicarboxylates are the dominant carbon source made available to the bacteroid as a source of energy and reductant for nitrogen fixation.

4. Carbon Metabolism via the TCA Cycle

The tricarboxylic acid (TCA) cycle is the dominant metabolic pathway by which C_4-dicarboxylates are oxidized in bacteroids (see Figure 1). Studies with radiolabelled metabolites have shown that the TCA cycle is present in *B. japonicum* (Keele et al., 1969, Tajima et al., 1990) and all TCA cycle enzyme activities have been observed in several *Rhizobium* species. (Saroso et al., 1986, Finan et al., 1988, McKay et al., 1989, Finan et al., 1991). Supporting genetic evidence has shown that mutants of *S. meliloti* lacking isocitrate dehydrogenase (McDermott and Kahn, 1992), α-ketoglutarate

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dehydrogenase (Duncan, Fraenkel, 1979), and succinate dehydrogenase (Gardiol et al., 1987) are unable to support nitrogen fixation. *B. japonicum* α-ketoglutarate dehydrogenase mutants can grow on a variety of carbon sources including malate and succinate indicating that at least part of the TCA cycle can be by-passed in this organism (Green, Emerich, 1997). Evidence that the glyoxylate shunt is functional in bacteroids is limited since isocitrate lyase activity during symbiosis is low (e.g. Green et al., 1989). It is therefore unclear if the entire TCA cycle is operating in *B . japonicum* bacteroids.

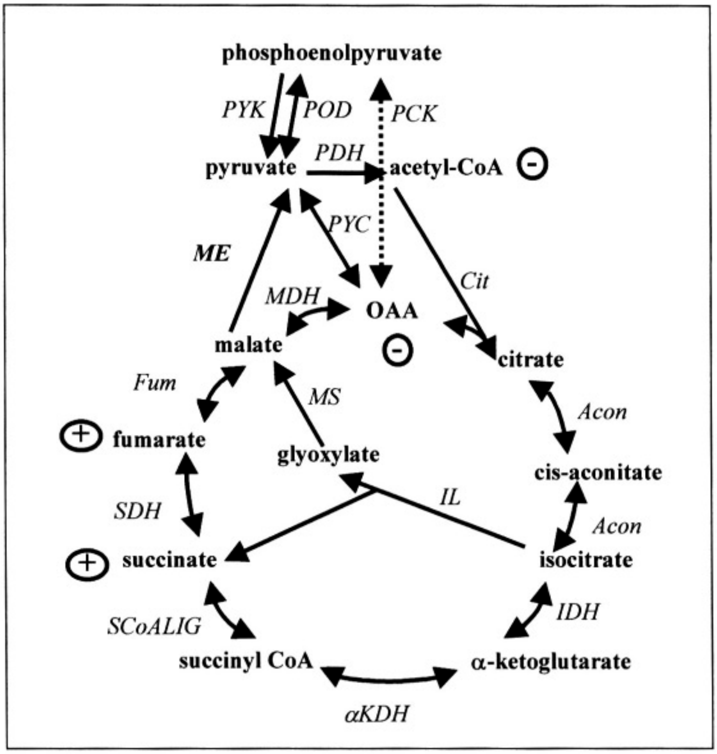
phosphoenolpyruvate
PYK
POD
PCK
PDH
pyruvate
acetyl-CoA
−
PYC
ME
Cit
MDH
OAA
malate
−
citrate
Fum
MS
Acon
+
fumarate
glyoxylate
cis-aconitate
IL
SDH
Acon
+
succinate
isocitrate
IDH
SCoALIG
succinyl CoA
α-ketoglutarate
αKDH

Figure 1. Tricarboxylic acid (TCA) cycle. *PYK*; pyruvate kinase, *POD*; pyruvate orthophosphate dikinase, *PCK*; phosphoenolpyruvate carboxykinase, *PDH*; pyruvate dehydrogenase, ***ME***; malic enzyme, *PYC*; pyruvate carboxylase, *Cit*; citrate synthase, *Acon*; aconitase, *IDH*; Isocitrate dehydrogenase, *αKDH*; α-ketoglutarate dehydrogenase, *MDH*, malate dehydrogenase; *SCoALIG*; succinyl CoA ligase, *SDH*; succinate dehydrogenase, *Fum*; fumarase. + and - refer to compounds effects on NAD-ME of *S. meliloti*.

5. TCA Cycle Maintenance Requirements

To maintain the TCA cycle with only C_4-dicarboxylates as the sole carbon source, a pathway to generate acetyl-CoA is essential. Several such enzymatic pathways exist but their relative importance to nitrogen fixation vary. Pyruvate can be produced from oxaloacetate by the action of pyruvate carboxylase (PYC) (see Figure 1), however, mutants of several *Rhizobium* species lacking this activity are able to fix nitrogen (Arwas et al., 1986; Ronson and Primrose, 1979; Dunn et al., 1996). Oxaloacetate decarboxylase, which converts OAA to pyruvate, has not been detected in *S. meliloti* (Duncan, Fraenkel, 1979) and so is not likely to play a role in symbiosis. Phosphoenolpyruvate carboxykinase (PCK), in association with pyruvate kinase could be used to produce pyruvate (see Figure 1) and Pck mutants vary in terms of their effects on nitrogen fixation. In *R. leguminosarum* Pck mutants induce Fix^+ nodules (McKay et al., 1985) while in *S. meliloti* Pck^- strains induce nodules which have 60% nitrogen fixation ability relative to wild type nodules (Finan et al., 1991). Interestingly the symbiotic phenotype of Pck mutants of the broad host range *Rhizobium* sp NGR234 is host dependent (Østerås et al., 1991). The latter may reflect differences in the carbon sources available to the bacteria during the infection process. Malic enzymes (ME) are capable of oxidative decarboxylation of malate to pyruvate with a concomitant reduction of a nicotinamide cofactor (see Figure 1). Since no other pathway leading from TCA cycle intermediates to pyruvate appears to be functional at a level capable of sustaining nitrogen fixation in the *Rhizobiaceae*, we believe that malic enzymes are the dominant means by which the TCA cycle is maintained in bacteroids.

6. Malic Enzymes Detected in *Rhizobium sp.*

Malic enzyme activity has been observed in several *Rhizobium* species including *S. meliloti*, *R. leguminosaurm*, *Rhizobium* NGR234, and *B. japonicum.* In *B. japonicum* two enzymes have been identified which differ in cofactor specificity (Copeland et al., 1989) and both enzymes have been partially purified and characterized at the biochemical level. The $NADP^+$ dependent ME has a high affinity for malate (Km 61 μM) and is inhibited by the TCA cycle intermediates acetyl-CoA and oxaloacetic acid (Chen et al., 1997) as well as citrate, malonate, and glyoxylate (Kimura and Tajima, 1989). The NAD^+ dependent ME showed a lower affinity for malate (Km 270 μM) and was activated by glycolytic intermediates such as D-glucose-6-phosphate, D-fructose-6-phosphate, and D-fructose-1,6-diphosphate and inhibited by fumarate (Chen et al., 1998). SDS-PAGE analysis revealed that both enzymes had subunit molecular weights of approximately 80 kDa with the holoenzyme exhibiting variable structure. The $NADP^+$-ME alternated between a dimer at pH 7 or a tetramer at pH 8 while the NAD^+-ME exhibited a tetrameric structure only. The genes encoding these ME's have not yet been reported so their respective importance to nitrogen fixation is unknown. In *R. leguminosarum* ME activity which utilizes both NAD^+ and $NADP^+$ has been identified (McKay et al., 1988) but no further information is available. Work in our laboratory has focused on the two ME's of *S. meliloti* and their importance in N_2-fixing bacteroids. Mutants deficient for ME activity have been isolated and their symbiotic phenotypes characterized. The genes encoding the diphosphopyridine dependent ME (DME) [EC 1.1.1.39] and the triphosphopyridine dependent ME (TME) [EC 1.1.1.40] genes have been isolated, overexpressed and the resulting enzymes purified and characterized.

7. *S. meliloti* Malic Enzyme Structure

The nucleotide sequence of the *dme* and *tme* genes revealed that the respective proteins have an unusual chimeric structure (see Figure 2) relative to previously described prokaryotic malic enzymes (Mitsch et al., 1998). Both polypeptides have a C-terminal extension of approximately 320 amino acids, which show similarity in sequence and length to phosphotransacetylase (PTA, EC 2.3.1.8.), an enzyme, which interconverts acetyl-phosphate and acetyl-CoA. With the expanded interest in genomics several other "extended" malic enzymes have been identified from *H. influenzae* (Fleishman et al., 1995), *E. coli* (Blattner et al, 1997) and *R. prowazekii* (Andersson et al., 1998), indicating that the *S. meliloti* ME's are members of a new class of prokaryotic malic enzymes. The native molecular weight of both DME and TME was determined to be approximately 620-680 kDa indicating an octameric structure based upon a subunit size of 82 kDa derived from the sequence information. Elimination of the C-terminal region by utilizing convenient restriction enzymes located between the malic enzyme and PTA-like domains (see Figure 2) has resulted in the production of truncated enzymes which retain ME activity (Mitsch et al., 1998). Truncated forms of both DME and TME were unable to oligomerize to octamers and only dimers were detected in non-denaturing gels.

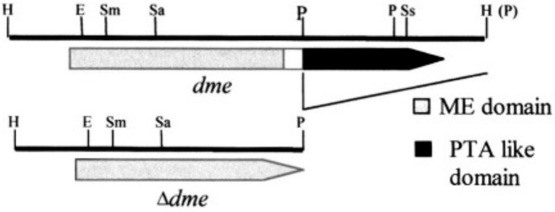

Figure 2. Schematic diagram of the intact and truncated NAD-ME of *S. meliloti* showing the chimeric nature of the gene. Restriction sites: E; *Eco*RI, H; *Hin*dIII, P; *Pst*I, Sa; *Sal*I, Sm; *Sma*I, Ss; *Sst*I.

8. Biochemical Characterization of *S. meiloti* Malic Enzymes

Both *dme* and *tme* were overexpressed in an *E. coli* background and their gene products were purified to homogeneity. The resulting proteins had subunit molecular weights of about 82 kDa as deduced from SDS PAGE supporting the predicted polypeptide molecular weights obtained from the sequence data. These *S. meliloti* proteins are therefore similar in size to the *B. japonicum* ME's and this suggests that the latter proteins also have a chimeric structure. Biochemical analysis of the two proteins revealed that DME exhibited positive cooperativity with malate but Michaelis-Menten type behavior with respect to the nicotinamide cofactors (Voegele et al., 1999) while TME showed Michaelis-Menten behavior for both substrate and cofactor. DME had a Km of 9.4 mM, 89 μM and 1.56mM for malate, NAD^+ and $NADP^+$ respectively. TME had a Km of 2.6mM for malate and 33 μM for the $NADP^+$ cofactor. The enzymes also showed substantial differences in terms of their response to TCA cycle intermediates. DME affinity for malate was stimulated by both succinate and fumarate and was inhibited by acetyl-CoA and OAA. This allosteric regulation is a novel mechanism for NAD^+-ME's but it does hint at the importance of this enzyme to the TCA cycle. DME seems able to detect increases in carbon flow to the TCA cycle via succinate and fumarate and increase activity to produce more pyruvate and therefore more acetyl-CoA for citrate production resulting in an increase of TCA cycle function. Conversely, an increase in acetyl-CoA concentration down regulates DME activity to ensure that more malate is utilized by MDH to maintain the TCA cycle balance. TME shows no allosteric regulation and shows only end-product inhibition and this lack of sensitivity to TCA cycle compounds may be significant in the bacteroid. To explore the significance of the C-terminal domain, assays to detect PTA activity were conducted but these revealed that no such activity was present (Voegele et al., 1999).

9. Symbiotic Requirements and Expression Patterns of *S. meliloti* Malic Enzymes

S. meliloti strains have been produced in which one or both malic enzyme genes have been eliminated. Strains lacking DME activity are able to form nodules but nitrogen fixation is lacking resulting in a Fix^- phenotype (Driscoll and Finan, 1993), while *tme* mutants are fully functional in regard to their nitrogen fixation ability (Driscoll and Finan, 1996). Therefore, in *S. meliloti*, DME is essential for nitrogen fixation while TME is not required. By assaying for DME and TME activity in free living cells grown under a variety of conditions it has been observed that neither enzyme is effected by succinate or glucose and both genes appear to be constitutively expressed (Driscoll and Finan, 1993). In bacteroids, however TME activity is reduced relative to that of free living cells while DME activity is unchanged. These observations were supported by *lacZ* fusion results which showed that *dme* transcription is unaltered on different carbon sources or in the bacteroid while *tme* expression falls to 20% relative to free living cells (Driscoll and Finan, 1997). From this data it is clear that *tme* is down-regulated in association with the plant host, possibly to eliminate anabolic processes that are not required in the senescent bacteroid.

10. Alterations to Malic Enzymes and Effects on the Symbiotic Phenotype

In order to explore the possibility that the level of TME activity in bacteroids is insufficient to maintain the TCA cycle, *tme* was placed under control of the *dme* promoter. In a *dme*$^-$ background, these constructs did not restore symbiotic N_2-fixation, despite the increased levels of TME present in bacteroids as detected by both Western blot and enzyme assays. It would seem that TME, despite having kinetic parameters similar to DME, it is not able to functionally replace DME during symbiosis. The reason for this may be that there is insufficient NADP present in the bacteroid for TME function or that TME is unable to interact with TCA cycle intermediates in the allosteric manner that DME does. In a related experiment, *dme* was placed under the control of the *tme* promoter and in a *dme*$^-$background, this construct gave plants that had approximately 20% the dry plant weight and acetylene reduction activity of plants inoculated with the wild type. This is consistent with our previous expression studies showing that *tme* is down-regulated in the bacteroid. To address the importance of the C-terminal region for nitrogen fixation, the truncated *dme* gene was recombined into the chromosome of several *dme*$^-$ *S. meliloti* strains. The resulting plant tests showed that the truncated gene was able to support symbiotic nitrogen fixation at levels comparable to wild type inoculated plants indicating that the PTA-like domain is not essential for bacteroid function. A tandem experiment was also carried out with the NAD^+ dependent ME of *E. coli* (Stols and Donnely, 1997). This ME (*sfcA*) has 21% identity and 32% similarity to the ME domain of DME and lacks the PTA like C-terminal extension. Phylogenetically, it is more closely related to eukaryotic rather than prokaryotic ME's. The *sfcA* gene was placed under the control of the *dme* promoter and inserted into a variety of *dme* and *tme S. meliloti* mutants. The symbiotic phenotype of the resulting strains revealed that the *E. coli* ME was functional in bacteroids and produced plants that had comparable nitrogenase activity and dry weights to wild type inoculated plants. These results support the conclusion that the PTA-like C-terminal domain is not essential for symbiotic nitrogen fixation and at present its function remains unknown.

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MOLECULAR BIOLOGY OF THE PERIBACTEROID MEMBRANE

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1. Introduction

The peribacteroid membrane (PBM) is a plant membrane that surrounds nitrogen-fixing rhizobia in the cells of legume root nodules. The PBM is formed initially via endocytosis of the plant plasma membrane as rhizobia enter nodule cortical cells (reviewed in Whitehead, Day, 1997). The resulting organelle that consists of PBM and the enclosed bacteria is called a symbiosome. The subsequent synthesis of PBM that accompanies bacterial proliferation relies on the endomembrane system which delivers both lipid and protein to the membrane. Some PBM proteins may also be inserted post-translationally from the cytoplasm (Simonsen, Rosendahl, 1999).

The PBM separates rhizobia from the plant cell cytoplasm and, therefore, is strategically placed to control nutrient and signal exchanges between the two symbionts. Biochemical and biophysical studies of the PBM have revealed the presence of a number of transporters that are likely to be key players in nutrient exchange between the host and its microsymbionts. These include a P-type H^+-ATPase that generates a proton-motive-force across the PBM, a dicarboxylate transporter that provides reduced carbon to bacteroids, and an ammonium transporter that exports ammonium from symbiosomes to the plant cytosol (reviewed in Udvardi, Day, 1997). The PBM also contains transporters for inorganic anions, iron, calcium, and auxin. In addition, a number of enzyme activities have been detected on the PBM, including a pyrophosphatase, a calcium-dependent protein kinase, and an iron chelate reductase (reviewed in Whitehead, Day, 1997). Importantly, the genes encoding each of these proteins remain to be identified. On the other hand, molecular genetic approaches have identified cDNA's encoding several PBM proteins, including nodulins 23, 24, and 26 (Jacobs et al., 1987). In general, the function of these proteins remains unknown.

To learn more about the biochemical and physiological roles of specific PBM proteins, we have used a number of complementary approaches to isolate cDNA encoding novel PBM proteins for functional studies. The approaches are based on functional homology, sequence homology, or proteomics. The first two approaches target genes that encode proteins with activities similar to those that have been measured on the PBM. For instance, we used yeast mutant complementation to identify a soybean nodule ammonium transporter with properties similar to those of the PBM ammonium transporter (Kaiser et al., 1998). An analagous approach is underway to isolate cDNA encoding calcium transporters. On the other hand, we have used a PCR-based approach to amplify conserved regions of cDNA (from both soybean and *Lotus japonicus)* encoding other transporters that are known to be located in the PBM. So far, cDNAs encoding P-type H^+-ATPases and putative iron transporters have been identified (Thomson et al., unpublished). EST sequencing and sequence analysis, in parallel with cDNA library screening using heterologous probes has yielded clones encoding two different ammonium transporters of the AMT family, one of which may be involved in PBM ammonium transport (Simon et al., unpublished). Invariably, however, approaches based on functional or sequence homology can only identify candidate PBM proteins. Other methods, such as immunolocalisation are required to confirm the intracellular location of these proteins. To circumvent this problem we have employed the tools of proteomics to isolate and sequence PBM

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proteins directly, as a prelude to cloning the corresponding cDNA. The results of this work are presented below.

2. Results and Discussion

Peribacteroid membrane was purified from nitrogen fixing root nodules, as described previously (Kaiser et al., 1998). Absence of cytochrome C reductase activity indicated that PBM preparations were free of bacteroid and mitochondrial membrane contamination. PBM proteins were separated by isolelectric focussing (IEF) and SDS-PAGE before being sequenced from the N-terminal end. Seventeen proteins were successfully sequenced, eight of which matched sequences of known proteins (Table 1).

Table 1. Homology of PBM proteins to known proteins

Protein	MW (kd)	Homologs	% Identity
2a/b	60	*Narcissus pseudonarcissus* HSP60 (AAB47206)	100% (15/15)
3	70	*Glycine max* BiP (AAA81956)	100% (15/15)
15	33	*Glycine max* Thiol protease (AAB16997)	73% (11/15)
18	80	*Arabidopsis thaliana* Subtilisin protease (AAD25747)	75% (9/12)
22	40	*Nicotiana tabacum* PDI (Y11209)	73% (11/15)
74	31	*Glycine max* Nodulin 53b (AF065435)	100% (11/11)
102	29	*Glycine max* Nodulin 26b (P08863)	92% (12/13)

Six of the putative PBM proteins are homologous to proteins of known function. These include three chaperones (HSP60, BiP (HSP70), and PDI) and two proteases (a serine and a thiol protease), which are all involved in some aspect of protein processing in plants. The PBM homologues of these proteins may play roles in protein translocation, folding, maturation, or degradation in symbiosomes. Two proteins are homologous to known nodule-specific proteins from soybean, nodulin 53b and nodulin 26B. Although the function of these nodulins is unknown, nodulin 53b has been independently shown to be associated with the PBM. All of the eight proteins with identifiable homologues are likely to be peripheral rather than integral membrane proteins, probably because of difficulties associated with solubilisation of hydrophobic proteins for IEF. The identification of homologues of HSP70 and HSP60 associated with the PBM is the first evidence that molecular machinery for co- or post-translational import of cytoplasmic proteins is present in symbiosomes. This supports recent studies on post-translational import of nodule proteins into isolated symbiosomes (Simonsen, Rosendahl, 1999), and has important implications for the biogenesis of this unique nitrogen-fixing organelle.

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OXYGEN REGULATION AND ADENYLATES IN LEGUME NODULES

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1. Introduction

In legume nodules, the average O_2 concentration in the bacteria-infected cells is so low (<60nM, Kuzma et al. 1993) that it limits nodule respiration and nitrogenase activity. By adjusting their permeability to O_2 diffusion, legume nodules exercise physiological control over their infected cell $[O_2]$ thereby up- or down-regulating nodule metabolism and N_2 fixation. This paper reviews three recent studies to provide some novel perspectives on (a) how the infected cell O_2 concentration is regulated in soybean, (b) what intracellular adenylate pools tell us about the site of O_2 limited metabolism in legume nodules (i.e. plant mitochondria or bacteroids), and (c) why the adenylate energy charge in legume nodules is so low.

2. The Regulation of O_2 Diffusion

Although the inner cortex of legume nodules has been proposed as the primary site for the regulation of O_2 permeability, it is questionable whether such a biophysical control system could have sufficient regulatory precision over infected cell $[O_2]$ (at least 0.1% ± 0.01% of $[O_2]$ in outer cortex) (Fig. 1, dashed line). Also, the low intercellular space O_2 concentrations in such a nodule would preclude having uninfected cells with an $[O_2]$ approaching the measured $Km(O_2)$ of uricase (30 μM, Rainbird and Atkins, 1981).

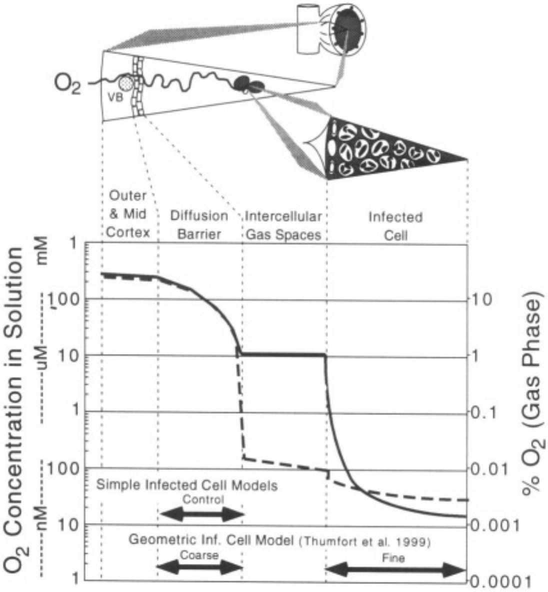

Fig. 1. Predicted O_2 gradients across a legume nodule. Predictions of Thumfort et al. (1999) (solid line) are compared with a model which assumes all control of O_2 diffusion is located in the inner cortex (dashed line).

A 3-D model of O_2 diffusion (Thumfort, et al. 1999) into infected and uninfected cells provided evidence in support of two stage O_2 regulation: coarse control by an inner cortical diffusion barrier and fine control through an innate, homeostatic control mechanism in the infected cells (Fig. 1). This model predicted that it may be possible for uninfected cells (the site of uricase) within the nodule to have an O_2 concentration 100 times higher (or more) than that in adjacent infected cells.

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3. The Site of O_2 Limited Metabolism.

The Thumfort model predicted high O_2 concentrations near the intercellular spaces where the mitochondria congregate **($Km(O_2)$ = 50-100 nM,** Millar et al. 1995), and much lower concentrations in the region of the bacteroids **($Km(O_2)$ = 20 nM,** Bergersen 1994), suggesting that the bacteroids, not the mitochondria, would be the site of O_2 limited metabolism in nodules.

This hypothesis was tested with a non-aqueous technique that quantified adenylate pool sizes in plant and bacteroid fractions of soybean nodules before and after imposition of an O_2 limitation (Kuzma et al. 1999). The observed decline in whole nodule adenylate energy charge (AEC = (ATP+0.5ADP)/(ATP+ ADP+AMP)) from 0.70 ± 0.01 to 0.61±0.04 was attributed to a decline in bacteroid AEC from 0.76±0.05 to 0.56±0.06. The plant AEC remained unchanged at 0.65±0.04, regardless as to whether the nodule was O_2 sufficient or O_2 limited.

These studies indicate that when legume nodules decrease their permeability to O_2 diffusion, they cut off the supply of O_2 to bacteroids, thereby restricting the ability of these bacteroids to metabolize carbohydrate and fix N_2.

4. Why the low AEC in the Nodule Plant Fraction

In aerobic tissues, AEC values are typically 0.80 or greater (Pradet & Raymond, 1983), values that are much higher than those found in the plant fraction of soybean nodules (0.65). If the plant fraction is not the site of O_2 limitation, why is the AEC so low? We (H. Wei and D.B. Layzell, unpublished) hypothesized that the low AEC may be explained by large ATP and ADP gradients in the infected cells as a result of (a) mitochondrial clustering near the intercellular spaces, (b) high cytoplasmic ATP demand near the symbiosomes (for glutamine synthetase activity and symbiosome transport) and (c) the large cell size (typically, diameter of **50-60 μm)** (Fig. 2).

To test this hypothesis, a mathematical model was built to describe cytosolic adenylate diffusion. The model predicts large adenylate gradients across an infected cell, resulting in an AEC gradient of 0.2 units or more (H. Wei and D.B. Layzell, unpublished). Assuming an AEC of >0.80 in the mitochondrial zone, the model readily accounts for the low AEC that has been observed in the plant fraction of legume nodules.

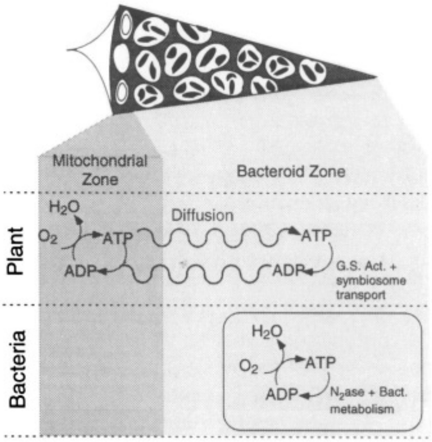

Fig. 2. A diagram showing the sites of ATP synthesis and utilization in nodules, and the diffusion pathways involved.

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THE *CBB$_3$*- AND THE PUTATIVE *BD*-TYPE TERMINAL OXIDASES OF *BRADYRHIZOBIUM JAPONICUM*

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1. Introduction

Bradyrhizobium japonicum can terminate aerobic respiration with several alternative oxidases. These oxidases are expressed under different environmental conditions and allow the organism to compete in various biological habitats. Intensive efforts have been made to investigate the ***cbb*$_3$-type** oxidase of *B. japonicum.* This oxidase is required for symbiotic nitrogen fixation in the soybean root nodules (*Glycine max*) (Preisig et al. 1993). It was previously demonstrated that the purified ***cbb*$_3$-type** oxidase has an extremely high affinity for oxygen ($K_M[O_2] \leq 7nM$; Preisig et al. 1996). Bacteroid respiration thus becomes possible under the low oxygen partial pressure in root nodules. Oxidative phosphorylation to form ATP is expected to be the biological source of energy to meet the high energy demand for nitrogen fixation. In order to find out whether the ***cbb*$_3$-type** oxidase can generate an electrochemical proton gradient, we performed a classical proton pump experiment. The ***cbb*$_3$-type** oxidase was reconstituted into soybean phospholipids, and experiments to show proton translocation were performed. In addition, we report here the discovery of genes (*cydAB*) encoding a putative terminal oxidase of the *bd* type. This is now the fifth terminal oxidase in the branched respiratory chain of *B. japonicum.* In some organisms, *bd*-type oxidases are known to be expressed under low oxygen concentrations. The *Azotobacter vinelandii bd*-type oxidase is responsible for respiratory protection, and in *Azorhizobium caulinodam* the ***cbb*$_3$-** and *bd*-type oxidases contribute about equally to symbiotic nitrogen fixation activity (Kelly et al. 1990; Kaminsky et al. 1996). To address whether in *B. japonicum* a *bd*-type oxidase is also involved in respiration under symbiotic conditions, we constructed a *cydA*::*aph* cassette insertion mutant and tested its fixing ability.

2. Material and Methods

The ***cbb*$_3$-type** oxidase of *B. japonicum* was purified (Zufferey et al 1998). The soybean phospholipids were purified as described by Kagawa et al. (1971). The oxidase and the phospholipids were mixed and incubated at 4°C, followed by dialysis at 4°C for about 60h to facilitate the formation of proteoliposomes. The reconstituted oxidase was characterized as follows. First, the proportion of right-side-out vesicles was determined by recording the oxidation of reduced horse heart cytochrome *c* by the reconstituted ***cbb*$_3$-type** oxidase. After 20 sec of oxidation, the proteoliposomes were solubilized with 0.1% dodecylmaltoside, and the increased oxidation was recorded. The quotient of the activities before over after addition of detergent gave the percentage of right-side-out vesicles. Second, the respiratory control ratio (RCR) (Ludwig, et al. 1980) was determined. Cytochrome *c* oxidation was recorded both in the absence of uncouplers of oxidative phosphorylation (valinomycin and CCCP) and in their presence. This assay demonstrated that two compartments are formed during dialysis, and charge translocation is established by the ***cbb*$_3$** oxidase. Finally, a proton pump experiment according to Solioz et al. (1981) was performed. Proteoliposomes were pulsed by the substrate (cytochrome *c*) in a thermostated vessel. At the same time, the pH of the surrounding medium was recorded. Upon a pulse of cytochrome *c*, an immediate

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decrease of the pH indicated that protons were extruded by the ***cbb*$_3$-type** oxidase.

3. Results

The proton pump activity of the ***cbb*$_3$** oxidase of *B. japonicum* was investigated. We observed after the reconstitution procedure a 70-80% right-side-out population of ***cbb*$_3$** oxidase vesicles. The RCR was about 5.8-6. This means that during the oxidation of cytochrome *c* a proton-motive force is established by the ***cbb*$_3$** oxidase. In the proton pump experiment we observed a small but reproducible proton pump activity between $0.3H^+/e^-$ and $0.5H^+/e^-$. We conclude that the ***cbb*$_3$-type** oxidase participates in the establishment of a proton-motive force, which then is used for ATP generation in symbiotic bacteroids.

In an unrelated sequencing project, a *B. japonicum* DNA region containing two genes with similarity to known *bd*-type oxidase genes was found. The amino acid sequence encoded by the *cydA* and *cydB* genes of *B. japonicum* revealed 40% identity with CydA and 39% identity to CydB of *Rickettsia prowazekii.* No typical regulatory sequences for transcription were found upstream of *cydA.* A gene encoding a putative ubiquinol-dependent malate:oxaloacetate oxidoreductase was found to be located upstream of *cydA.* It is possible that this gene is cotranscribed with *cydAB.* A *cydA*::*aph* insertion cassette mutant was constructed and found to behave normally in nodulation and symbiotic nitrogen fixation. Furthermore, did not have any growth defect under various aerobic and anaerobic growth conditions. In order to find expression conditions for this new oxidase, a *cydA'*- *'lacZ* fusion was constructed. **β-Galactosidase** activity was observed on plates with minimal medium supplemented with 0.3% malate and 0.3% lactate. This oxidase is apparently expressed at a very low level. We suggest that the *bd*-type oxidase of *B. japonicum* is an additional terminal ubiquinol oxidase that terminates respiration under so far unknown environmental conditions.

4. Discussion

We have demonstrated that the ***cbb*$_3$-type** oxidase, which is essential for nitrogen fixation, can couple electron flow to the generation of an electrochemical proton gradient. The fact that pump activity was below $1H^+/e^-$ could be due to the use of horse heart cytochrome *c* as an artificial electron donor. It will now be interesting to use the probable natural electron donor for the ***cbb*$_3$-type** oxidase, the cytochrome ***bc*$_1$** complex. We demonstrated here for the first time that a rhizobial oxidase with extremely high affinity for oxygen can function as a proton pump.

We have also found the *cydA*- and *cydB-homologous* genes encoding a fifth terminal oxidase in *B. japonicum.* It is putative a *bd*-type ubiquinol oxidase. Based on the Fix^+ phenotype of a *cydA*::*aph* mutant, we can exclude that this putative *bd*-type oxidase is important for nitrogen fixation. This is different in at least two aerobic diazotrophs (Kelly et al. 1990, Kaminsky et al 1996). However, the optimal conditions for expression of this oxidase remain to be elucidated.

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AMMONIUM AND ALANINE ARE THE PRIMARY NITROGEN SECRETION PRODUCTS OF PEA BACTEROIDS

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It has long been considered that the nitrogen excretion product of legume bacteroids is ammonium, with little nitrogen assimilation occurring inside bacteroids. However, labelling data for soybeans and peas indicated substantial synthesis of both glutamate and alanine (Salminen & Streeter, 1992). Alanine in particular was rapidly labelled and appeared to turn over, unlike the rather inert glutamate pool. Further support for alanine synthesis came from direct measurement of alanine synthesis and secretion by pea peribacteroid units (Rosendahl *et al.,* 1992). In a direct challenge to the established model that ammonium is the primary secretion product of nitrogen fixation it was shown recently that soybean bacteroids are capable of secreting only alanine, without ammonium, when supplied with $^{15}N_2$ (Waters *et al,* 1998).

In an attempt to understand these profound contradictions we begin a joint biochemical and genetic study of the pathways and importance of alanine secretion in pea bacteroids. The first part of this study was to examine the kinetic constants of alanine synthesis. To do this pea bacteroids were purified rapidly under argon by Percoll gradient centrifugation. Bacteroids were then incubated in a closed assay system with L-malate and N_2 at various O_2 tensions and the nitrogen secretion products measured. The bacteroids were able to reduce N2 to ammonium at high rates, with an optimal rate at an O_2 tension of 1%. This rate was reduced by 60% at 0.1% O_2 and was inactivated by 5% O_2. At low-moderate bacteroid density only ammonium could be detected as a secretion product. However, when the bacteroid density was high (equal to or greater than 10 mg/ml protein), ammonium and alanine were made rapidly. We considered this might be due to the build up of ammonium in the fixed volume of the assay activating alanine synthesis. This was demonstrated by incubating low density bacteroid preparations in exogenous ammonium. Alanine synthesis began immediately if ammonium was present. The whole cell K_m for alanine synthesis is 3.2 mM NH_4^+, similar to the K_m of alanine dehydrogenase (5.1 mM). Such ammonium concentrations are expected to be present in legume nodules; it has been estimated to be 12 mM in soybean bacteroids (Streeter, 1989). In a parallel study we isolated a large number of transposon mutants unable to grow on alanine as the sole carbon source. Mutants were identified in a putative alanine transport system and in the *dad* operon. The products of the *dad* operon, DadR (LRP like regulator), DadX (alanine racemase) and DadA (an electron transport chain linked D-alanine dehydrogenase, not to be confused with NAD^+ dependent L-alanine dehydrogenase) are responsible for the isomerisation of L-alanine to D-alanine and its subsequent oxidation. The Dad operon is the main route for alanine catabolism in *R. leguminosarum.* Our *dadR* mutant, strain RU1275, was complemented by a cosmid pRU3131 that contains the *dad* operon and was suppressed by an unrelated cosmid pRU3135. The cosmid pRU3135 was subjected to saturation mutagenesis with Tn5-*lacZ* and a cosmid isolated that no longer suppressed the *dadR* mutation in strain RU1275. Sequencing showed that the transposon had mutated alanine dehydrogenase (*aldA*). AldA is still present in strain RU1275 but will only suppress the growth defect on alanine as the sole carbon source when present in multiple copies. This suggested that the main role of AldA is not alanine catabolism. This was demonstrated by making a chromosomal mutant of *aldA* (strain RU1327), which still grew normally on alanine as the sole carbon source.

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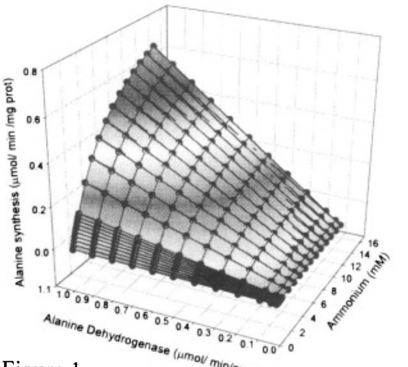
Strain RU1327 was inoculated onto peas and bacteroids isolated to examine the effect on nitrogen fixation and the nature of the nitrogen secretion products. Synthesis of alanine was absolutely dependent on alanine dehydrogenase since pea bacteroids mutated in *adh* (strain RU1327) did not secrete alanine. This confirmed that AldA is primarily an anabolic enzyme. No AldA activity was present in RU1327 while the wildtype had 0.09 μmol min^{-1} (mg protein)$^{-1}$, demonstrating there is only one active *aldA* gene in *R. leguminosarum.* Peas inoculated with RU1327 (*adh::*Tn5-*lacZ*) still fixed nitrogen as measured by acetylene reduction but their symbiotic performance was impaired. Peas inoculated with RU1327 and harvested at 6 weeks had a 20% decrease in shoot dry weight compared to the wildtype strain. These data show that pea bacteroids secrete both ammonium and alanine as primary nitrogen sources to the plant.

Given our work on the role of alanine synthesis and secretion by pea bacteroids we can understand many of the contradictions in the earlier literature. Alanine secretion (whole cell K_m for ammonium 3.2 mM) may be overlooked when insufficient ammonium has accumulated to activate AldA (K_m 5.1 mM). This will be a particular problem in open flow cell assays of bacteroids where the ammonium produced is continuously removed. It may also lead to overestimation of the steady state rate of alanine synthesis if the bacteroid density is very high in a closed assay system. Since the precise partitioning of ammonium to alanine can be altered so much by the *in vitro* assay conditions it is essential to isolate an *aldA* mutant before the importance of alanine synthesis *in planta* can be assessed. Assuming a constant affinity for ammonium by AldA there are two key variables that will alter the partitioning to alanine in any given legume species, these are the steady state ammonium concentration and the specific activity of AldA. The activity plane for AldA activity (K_m 5.1 mM) as a function of the steady state ammonium level and the specific activity is shown in Figure 1. Even at a steady state ammonium level of 0.5 mM and specific AldA activity of 0.09 μmol min^{-1} (mg protein)$^{-1}$ (the specific activity measured for pea bacteroids), the theoretical rate of alanine synthesis is 482 nmoles alanine h^{-1} (mg protein)$^{-1}$. This is 5-10 times the measured rate of ammonium and alanine secretion of pea bacteroids combined. At very high specific activities of AldA the rate of synthesis of alanine increases substantially over this. The activity of AldA in soybean bacteroids has been measured between 0.3-1 μmol min^{-1} (mg protein)$^{-1}$ suggesting soybean bacteroids may have an even greater partitioning to alanine than pea bacteroids. We conclude there is a plastic partitioning of the products of N_2 fixation to ammonium and alanine.



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REGULATION OF AMMONIUM ASSIMILATION DURING SYMBIOSOME DIFFERENTIATION IN ROOT NODULES OF *RHIZOBIUM ETLI*

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1. Introduction

During development of root nodules, *Rhizobium* bacteria are released endocytotically inside the invaded plant cells where they differentiate into their endosymbiotic form called symbiosomes (SBs). SBs surrounded by a plant-derived peribacteroid membrane are non-dividing, organelle-like structures, that use nitrogenase to reduce N_2 to NH_3. We are studying the molecular mecanism by which SBs, unlike free-living diazotrophs, are able to fix nitrogen (N) but unable to assimilate most the NH_4^+ produced therein. In fact, most of the N-fixed in SBs is liberated to the host plant to satisfy its demands for N, thus N-fixation and N-assimilation are expected to be uncoupled in SBs. However the form (e.g. NH_4^+ or organic compounds, such as amino acids) and the mechanism (passive diffusion or active transport) by which nitrogen is exported are still largely unclear. In N-fixing SBs the NH_4^+ transport (uptake) and assimilation (glutamine synthetase) activities are either absent or are expressed at a very low level. Consequently, the existence of a regulatory mechanism(s) by which genes involved in NH_4^+ assimilation are switched-off during bacteroid differentiation (before N-fixation commences) was speculated.

In *R. etli* transcription of genes involved in NH_4^+ assimilation, such as *amtB* (($NH_4^+/CH_3NH_3^+$ transport) and *glnII* (glutamine synthetase II, GSII), is controlled in response to nitrogen availability, by the global nitrogen regulatory system (*ntr* system) including the protein kinase NtrB and the nitrogen transcriptional regulator NtrC. Moreover, the DNA binding ability of the NtrC protein but not its intracellular concentrations, is influenced by the nitrogen availability. Furthermore, the NtrC protein disappears from bacteroids during their differentiation, by a three-step mechanism of gene regulation acting first by inactivation of the NtrC protein, then by down-regulation of *ntrBC* transcription occurring in bacteroids of young symbiosomes, and finally by the complete removing of NtrC protein. NtrC-dependent promoters, such as those controlling the expression of AmtB and GSII, are active in bacteria growing inside infection threads and in younger bacteroids, but are inactive in differentiated SBs; thus indicating that alternative transcriptional activator for those promoters are not present in SBs.

Therefore, inactivation of *ntr* system may be the first step of a mechanism acting during nodule invasion to uncouple nitrogen fixation and NH_4^+ assimilation activities. However some aspects of this mechanism remains unresolved: first, the signal transduction pathway acting to inactivate NtrC in SBs is unknown but in free-living bacteria NtrC is inactivated under nitrogen-sufficient conditions. Second, the mechanism by which the *ntrBC* promoter is switched off in SBs is presently unknown, but we have demonstrated that the *ntrBC*p is active only in dividing bacteria, thus the arrest of bacterial division occurring during SB differentiation may be correlated with the down-regulation of *ntrBC* transcription. Third, the molecular mechanism acting to remove the NtrC protein from SBs is presently unknown but other proteins are also removed from SBs during their differentiation. We

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observe that In *R. etli* protein degradation is enhanced under N-starvation, thus suggesting that SB should be N-starved, at least up to the activation of the nitrogenase complex.

2. Ectopic expression of *R. etli amtB* gene affects the SB differentiation process

It has previously been proposed that NH_3 produced by the bacteroidal nitrogenase passively diffuses across the bacteroid membrane into the relatively acidic peribacteroidal space (PBS) where it is converted to NH_4^-, which then enters the plant cytosol through a channel located on the peribacteroid membrane. Based on this model, the function and capacity of the symbiosome to export NH_4^+ depends on the absence of an NH_4^+ uptake system in the bacteroid membrane. Accordingly with this model, we observe that the *amtB* promoter is switched off during nodule invasion, just when bacteria are released inside the invaded plant cells. The lack of *amtB* expression could explain the absence of NH_4^+ uptake activity measured in isolated symbiosomes. We hypothesize that forcing the expression of the *amtB* gene in N_2-fixing bacteroids might help to confirm this model. We have shown that the ectopic expression of *amtB* in bacteroids alters the ability of bacteria to invade the host cells and the symbiosome differentiation process. The expression of *amtB* was also tested in different genetic backgrounds. Both the NtrC protein, which controls the expression of the bacterial genes involved in NH_4^+ assimilation, and the nitrogenase activity (NH_4^+ production) are essential to observe the *amtB*-mediated effect. We propose that the marked change in the process of nodule invasion and bacteroid differentiation, resulting from the ectopic expression of *amtB* can be explained considering that ions are retrieved from the PBS, thus generating an energy-consuming futile cycle and/or lowering the energized state of the bacteroid membrane. Our data support the idea that *in vivo* the bacteroids release NH_4^+ and that this event, occuring when the first invaded host cells contain only few symbiosomes, acts as a developmental signal controlling later steps of nodule invasion.

One of the earliest and most remarkable consequences of the ectopic expression of *amtB* during symbiosis (simultaneous induction of *amtB* and *nif* genes (nitrogen fixation) is the presence, in young nodules, of a reduced number of invaded cells containing only a few undiferentiated SBs. Since the *amtB* gene was expressed from a NifA-dependent promoter, this low number of undifferentiated symbiosomes indicates that the induction of *nif* genes occurs at a very early stage of nodule invasion. In fact, when a p*nifHc-lacZ* fusion was tested, it was observed that *pnifHc* is activated early during the invasion process, most probably when bacteria are released inside the invaded plant cells.

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THE ROLE OF RHIZOBITOXINE IN NODULATION BY *BRADYRHIZOBIUM ELKANII*

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1. Biochemical Characteristics of Soybean Bradyrhizobia

Bradyrhizobium elkanii was proposed as DNA homology group II of soybean bradyrhizobia, where five traits were listed to delineate *B. elkanii* and *B. japonicum:* extracellular polysaccharide composition; *ex plant* nitrogenase expression; major terminal oxidase, fatty acid composition and intrinsic antibiotic resistance (Kuykendall et al, 1992). However, rhizobitoxine, indole-3-acetic acid (IAA) and nitrite reduction should be mounted to the criteria to distinguish the both species of soybean bradyrhizobia as well, because these phenotypes were strictly correlated with them and are easily assayed. The differences between both species extended to host preference: *B. elkanii* and *B. japonicum* preferentially nodulated siratro (*Macroptilium atropurpureum*) and soybean, respectively (Minamisawa et al, 1997). The legume symbiont *B. elkanii* and the plant pathogen *Burkholderia andropogonis* synthesize rhizobitoxine [2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid], which causes foliar chlorosis on a variety of plants. Although rhizobitoxine synthesis seems to involve in pathogenicity in these plant-microbe interactions, little is known about positive role of rhizobitoxine in symbiosis.

2. Inhibition of ACC Synthase by Rhizobitoxine

Biochemical works have indicated that rhizobitoxine inhibit β-cystathionase in methionine synthesis pathway in bacteria and plants (Xiong K et al 1996). On the other hand, Owens et al. (1972) showed that rhizobitoxine inhibits the production of ethylene by measuring ^{14}C incorporation from methionine into ethylene. Yasuta et al (1998) demonstrated that rhizobitoxine competitively inhibits 1-aminocyclopropane-1-carboxylate (ACC) synthase bLE-ACS2 with a very low inhibition constant (25 nM). These findings indicate that ACC synthase is a target enzyme of rhizobitoxine, which bring about the inhibition of ethylene synthesis because ACC synthase is the rate-limiting enzymes in the biosynthesis of ethylene from methionine in higher plants.

3. Rhizobitoxine Synthesis in Soybean Bradyrhizobia

Ruan et al. (1993) isolated *rxtA* gene from *B. elkanii* USDA61, which is responsible for rhizobitoxine synthesis. When the DNA sequence of the portion around *rtxA* gene of *B. elkanii* USDA94, a stronger producer of rhizobitoxine, additional tree ORFs (putative *rtxC, rtxD* and *rtxE*) that were probably involved in rhizobitoxine production was found around *rxtA* gene. Indeed, the interruption of these ORFs aborted rhizobitoxine production of *B. elkanii* USDA94. The sequence homology of amino acid residues of the ORFs suggested that serinol (2-amino-1,3-propanediol) and gulutamate may participate in rhizobitoxine synthesis. Interestingly, *rtxA, rtxC* and *rtxD* were found in the symbiosis island of *B. japonicum* USDA110 spec (M. Gottfert, personal communication), although this strain does not excrete rhizobitoxine in culture and nodules. The presence of rhizobitoxine synthesis genes in *B. japonicum* suggests evolutionally significance of rhizobitoxine production in the establishments of (*Brady*)*rhizobium*-legume symbiosis.

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4. Enhancement of Nodulation and Competition by Rhizobitoxine

Exogenous application of ethylene generally blocks nodule formation in legumes (Lee et al, 1992). Moreover, rhizobitoxine inhibits ACC synthase, rate-limiting step for ethylene biosynthesis, as described above. Therefore, we hypothesized that rhizobitoxine enhance nodulation and its competitiveness under multistrain environments. We used siratro (*Macroputilium atropurpurem*) as a host plant, because *B. elkanii* preferentially nodulates this legume (Minamisawa et al, 1997). Addition of ACC, an ethylene precursor, reduced nodule formation in siratro roots with *B. elkanii* strain USDA94. To determine whether rhizobitoxine acts as a positive factor for nodulation, a rhizobitoxine production-deficient mutant RTS2 and its parent strains were compared. Ethylene emission in siratro roots inoculated with the mutant RTS2 was higher than that of the parent strain. The nodule number in siratro roots with RTS2 was slightly but significantly lower than that of the parent strain. Recently, similar reduction of nodule number of mungbean (*Vigna radiata*) was observed by rhizobitoxine mutant strains derived from *B. elkanii* USDA 61 (Duodu S, in press). In addition, rhizobitoxine production was involved in nodulation competitiveness; RTS2 showed significantly lower nodule occupancy than that of the parent strain in competition against the parent strain USDA94 of *B. elkanii* and against rhizobitoxine-nonproducing *B. japonicum* USDA110. These results suggest that rhizobitoxine enhances intra- and inter-species competitiveness of *B. elkanii* for siratro nodulation, which is probably brought about by the inhibition of ethylene synthesis in the root of host plant.

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POLYHYDROXYBUTYRATE IN NITROGEN-FIXING SYMBIOSES

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Polyhydroxybutyrate (PHB) accumulates in large amounts in free-living and bacteroid cells of many species of rhizobia, but the significance of this reserve material for nitrogen fixation is still unclear. PHB has been suggested to provide reserves of carbon and reductant that are important in sustaining respiratory activities to protect nitrogenase from damage by O_2, and in extending nitrogen fixation into the pod-filling stage (Romanov et al. 1980, Bergersen et al. 1991). On the other hand, there is evidence that suggests that, at least in some species of bacteroids, PHB reserves are not important for nitrogen fixation, and may actually reduce its capacity. Bacteroids in some symbioses (eg, chickpea, alfalfa) do not accumulate PHB, and nodules formed with *Rhizobium* mutants unable to synthesise PHB exhibit increased nitrogen-fixing activity (Povolo et al. 1994, Cevallos et al. 1996). Indeed, seeds from *Phaseolus vulgaris* plants nodulated by one such *R. etli* mutant contained significantly more nitrogen than seeds from plants nodulated with the wild type strain (Cevallos et al. 1996). Thus, questions remain as to whether carbon is allocated actively to PHB synthesis, or whether PHB synthesis is the result of an overflow pathway that operates because oxidation of carbon in the tricarboxylic acid cycle of bacteroids is constrained (Walshaw et al. 1997). To increase our understanding of the role of PHB symbiotic nitrogen fixation, we have been studying factors that influence the accumulation and mobilisation of this reserve material in several species of rhizobia. These studies are also aimed at determining whether accumulated PHB reserves enhance the survival of rhizobia during storage in peat cultures and after inoculation on to seeds.

Studies with *Mesorhizobium ciceri* CC 1192 have shown that PHB accumulates in cells that are grown in broth cultures, but not when these cells form bacteroids in root nodules on chickpea (*Cicer arietinum* L.) plants. Chickpea bacteroids have the enzymic capacity to synthesise PHB, which indicates that metabolic controls preferentially direct acetyl-CoA into the tricarboxylic acid cycle rather than towards PHB synthesis (Kim and Copeland 1996, Kim and Copeland 1997, Chohan and Copeland 1998). Studies of the enzymes concerned indicate that PHB synthesis will be favoured by high ratios of acetyl-CoA/CoA and $NADPH/NADP^+$. These ratios are likely to be influenced by changes in the environment in bacteroids.

In studies with *Rhizobium* sp. (*Lotus*) SU343, PHB granules were found to be abundant in broth-cultured cells in the late log phase of growth in a shaking incubator. With continued shaking, the PHB that had accumulated in these cells was completely mobilised within 2 days. The number of viable cells in these cultures doubled and then declined by 8 days to a value that was approximately one fifth of the original number. In contrast, when late log-phase cells were maintained without shaking, the number of viable cells in the cultures did not change and their PHB content remained the same for at least 2 weeks. These results suggest that (i) PHB can accumulate as a transient carbon reserve that may be remobilised to support cell proliferation, and (ii) that PHB reserves may enhance the survival of free-living cells. However, when SU343 cells were inoculated from the

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broth cultures on to plastic beads, the rate of survival was equally poor for cells that contained PHB or that had had their PHB reserves depleted. Thus, the presence of PHB does not seem to increase the fitness of the rhizobia for survival against the stresses that occur during inoculation. Examination of peat cultures of several species of rhizobia by electron microscopy indicated that a time-dependent thickening of the cell wall that occurred during storage may be associated with enhanced survival of cells after inoculation on to seeds.

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CHARACTERIZATION OF AN ACYL COENZYME A CARBOXYLASE FROM *RHIZOBIUM ETLI*

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Biotin, as the prosthetic group of biotin-dependent carboxylases, is essential for all organisms. To further define biotin-dependent metabolic pathways in *Rhizobium* (Encarnación et al 1995; Dunn et al 1996, 1997; Dunn 1998), we are characterizing an acyl CoA carboxylase from *R. etli* CE3. This enzyme was previously shown to be capable of carboxylating propionyl CoA (to form methylmalonyl CoA) and acetyl CoA (to form malonyl CoA; Dunn et al 1998) The acyl CoA carboxylase was purified 123-fold by a combination of chromatographic steps. The partially purified enzyme displayed its highest activity using propionyl CoA (C3:0) as a substrate, but was also able to carboxylate acetyl CoA (C2:0) and butyryl CoA (C4:0) at much lower rates. The apparent K_m and V_{max} values of the enzyme for propionyl CoA were 0.05 mM and 1448 nmol/min/mg protein, respectively, and the corresponding values using acetyl CoA as a substrate were 0.63 mM and 43 nmol/min/mg protein. These data show that the enzyme has a strong kinetic preference for propionyl CoA. The K_m values for ATP and bicarbonate were 0.09 and 0.71 mM, respectively, with propionyl CoA as a substrate. Of several potential effectors tested, compounds producing significant inhibition of the propionyl CoA or acetyl CoA carboxylating activities of the acyl CoA carboxylase included ATP, AMP, palmitate, stearate, valerate, methylmalonyl CoA and malonyl CoA. KC1 markedly activated the enzyme Analysis of the enzymes subunit and total molecular mass indicate an $\alpha 6 \beta 6$ structure. The α subunit (78 kDa) contains the biotin moiety. Although the kinetic preference of the acyl CoA carboxylase is for propionyl CoA, and may serve as part of an anaplerotic pathway using this substrate (Dunn 1998), we failed to detect a separate acetyl CoA carboxylating activity in *R. etli* apart from that catalyzed by the acyl CoA carboxylase. This indicates that the acyl CoA carboxylase, using acetyl CoA as a substrate, may be the sole enzyme responsible for catalyzing the first step in fatty acid synthesis, and thus play an indispensible role in the cell.

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RHIZOBITOXINE PRODUCTION OF *BRADYRHIZOBIUM ELKANII* ENHANCES SIRATRO NODULE FORMATION

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Rhizobitoxine (RT) , a *Bradyrhizobium elkanii*-producing ethylene synthesis inhibitor, has been thought as a potent factor to enhance nodule formation, since ethylene blocks nodule formation in some legumes. Addition of 1-aminocyclopropyl-l-carboxylic acid, an ethylene precursor, reduced nodule formation in siratro (*Macroptilium atropurpureum)* roots with *B. elkanii* USDA94. A RT-production deficient mutant RTS2 was constructed from USDA94 by homologous recombination with a RT-production related gene *rtxA*. Ethylene evolution in siratro roots with the mutant RTS2 was higher than that of the wildtype. The nodule formation in siratro roots with RTS2 was lower than that of the wildtype. The RT-production deficient mutant showed lower nodule occupancy than that of the wildtype in competition with RT nonproducing species *B. japonicum* USDA110. These results suggest that RT production of *B. elkanii* has roles for inhibition of ethylene synthesis and enhancement of nodule formation in siratro.

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THE ADENYLATE CYCLASE GENES OF *RHIZOBIUM ETLI*

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Cyclic adenine-mono-phosphate (cAMP) has been involved in several functions in *Rhizobia* like catabolic repression, the control of ammonium assimilation and hydrogen metabolism. However, the exact role of this nucleotide has not been elucidated mainly due to the absence of mutant strains defective in cAMP synthesis. The enzyme responsible for cAMP synthesis is the adenylate cyclase (AC), which is encoded by the *cya* gene. Here we present the molecular characterization of three different *cya* genes of *R. etli* isolated by complementation of a *cya Escherichia coli* mutant. The three different genes restore AC-dependent phenotypes like sensitivity to serine and phosphomycin as well as motility. Also, the complemented *E. coli cya* strains regained cAMP synthesis and accumulation. All three cya genes were located into the *R. etli* chromosome The nucleotide sequence of these genes was obtained and the ORF's responsible of the AC activity were identified. Two of them belong to the Universal Class of AC (Prog. Nucl. Acid Res. and Mol. Biol., 1994, 49: 241-283), while the third one did not match against any published sequence, probably representing a novel class of AC's. Each gene was disrupted with interposons and recombined back into the *R. etli* genome. Single mutants did not present any discernible phenotype in free life. We have already isolated a double mutant and are currently working in to generation of a triple mutant.

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ANALYSIS OF RHIZOBIAL TREHALOSE-6-PHOSPHATE SYNTHASE AND ITS ROLE IN ROOT NODULE DEVELOPMENT

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The non-reducing disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glycopyranoside) is widespread in lower organisms and is thought to be a protectant during stress conditions such as drought and heat. Trehalose is also present in plant roots during interactions with antagonistic fungi, mycorrhizal fungi, and in nitrogen-fixing root nodules (Müller et al. 1995). It is synthesised from UDP-glucose and glucose-6-phosphate in a two step process by the action of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. Homologues of the genes coding for these two enzymes in *E.coli, otsA* and *otsB,* have been located on the symbiotic plasmid (pNGR234*a*) of *Rhizobium* sp. NGR234 (Freiberg et al., 1997). To study the significance of rhizobial trehalose synthesis in nodule physiology, an Ω-cassette was inserted into the *otsA* homologue. In liquid cultures, trehalose biosynthesis was induced under low oxygen in NGR234. In its sym-plasmid free derivative ANU265 and in the mutant strain NGR234Δ*otsA* trehalose biosynthesis was impaired. In *Rhizobium* sp. NGR234 trehalose biosynthesis under low oxygen conditions is therefore directed by the sym-plasmid encoded *otsA* homologous gene.

To study the role of trehalose-6-phosphate synthase in root nodule development, various host plants were infected with *Rhizobium* sp NGR234Δ*otsA*. In an number of hosts, nodule number was increased and average nodule size reduced when compared to control plants infected with the wildtype strain NGR234. Analysis of carbohydrate content of these nodules revealed significant increases of sucrose and hexoses, indicating a potential role of this *otsA* homologue in nodule development and carbohydrate partitioning.

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CHARACTERIZATION OF *Rhizobium etli* relA/spoT HOMOLOGOUS GENE MUTANT

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1. Introduction

Most prokaryotes have the ability to respond to the lack of amino acids, carbon or energy sources by reorientating the cellular metabolism in a process known as the “stringent response.” In *Escherichia coli* this response is mediated by the accumulation of guanosine polyphosphate (guanosine 5’,3’ bis-diphosphate or ppGpp) by two pathways: one is a RelA enzyme and ribosome dependent pathway that synthetizes ppGpp when the cells are in amino acid starvation and the other is a SpoT enzyme dependent pathway during the carbon or energy starvation. The stringent response is a pleiotropic response that -between other changes in the cellular physiology- reduces the synthesis of stable RNA, modificates the gene expression and induces the stationary phase sigma factor in a preparatory process for surviving prolonged starvation. Our knowlegde of the rhizobia stringent response system is scarcely growing; not all the studied rhizobia elicit a classical stringent response: Sinor*hizobium meliloti* 1021 strain accumulates ppGpp following amino acid starvation but *Sinorhizobiwn meliloti* 41 strain does not (B. Belitsky, B. Kary, 1982), following an amino acid deprivation *Azorhizobium caulinodans* ORS571 accumulates ppGpp but the free living-fixing bacteria *Azotobacter vinelandii* and *Azomonas agilis* do not (S. Howorth, R. England. 1999). In the free-living nitrogen-fixing *K. pneumoniae* the stringent response is important for the nitrogen fixation; a relA mutant of this bacteria is unable to fix atmospheric dinitrogen after the soluble nitrogen has been exhausted from the media (Riesenberg, et. al. 1982).

2. Procedure.

We isolated a *R. etli* Tn-5 induced mutant unable to grow on minimal medium with glutamine as a sole carbon and nitrogen source. When we began the characterization of this mutant, we found a pleiotropic phenotype. The LM01 mutant is unable to grow on minimal medium with succinate and any of these amino acids (aspartic acid, glutamic acid or alanine as nitrogen source). It is unable to use nitrate as nitrogen source and cannot use glutamine, glutamic acid or alanine as a sole carbon and nitrogen source. The LM01 mutant poorly nodulates bean plants and this small nodules were unable to reduce acetylene. We cloned the genomic region of LM01 mutant that has the Tn5 insertion and sequenced the interrupted sequence, at the same time we sequenced the pMGD4.3 cosmid that complements all the LM01 mutant phenotype. The interrupted sequence in the LM01 mutant has an open reading frame that codes for a 744 amino acid protein quite homologous to a RelA protein of *M. xanthus* and a family of RelA and SpoT proteins of several bacteria. The stringent response is necessary for the utilization of some nitrogen and carbon sources, and for the symbiotic life in *R. etli.*

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METHIONINE IMPLICATION IN RHIZOBIAL IRON ACQUISITION FROM HEMOGLOBIN

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Iron is an essential metal for almost all living cells. In the interaction host-parasite, this metal is considered as a key element. The possession of highly efficient mechanisms to overcame the natural iron limitation imposed by the host, is critical for the parasite survival. Some parasite use siderophore to strip iron from the carrier proteins while others have evolved heme-mediated iron acquisition mechanisms. Although these mechanisms are complex and diverse, the presence of outer membrane receptors for iron compounds seems to be a general phenomenon. Despite the expanded understanding on the association rhizobia-legume, the knowledge of iron acquisition mechanism is one of the major interests of our group. We almost demonstrated that rhizobia were able to utilize hemin, hemoglobin, mioglobin leghemoglobin and siderophores (rhizobactin 242 and ferrichrome) as iron sources. Moreover, the uptake mechanism of these compounds share at least one component. According our previous work, it is conceivable that this component could be a protein from the Ton complex. These data support the idea that many of the processes found in the interaction rhizobia-leguminous host, are variant of general processes implicated in other micro- and macro- organisms interactions.

The present work aimed at the elucidation of the physiology and biochemistry of iron transport in rhizobia. In this study we examined the utilization of heme compounds and siderophores as iron sources in *Sinorhizobium meliloti* 242 grown on minimal medium.

Results obtained show that:

1- Sm 242 is unable to use heme-compounds as iron sources on iron-limited Vincent minimal medium (MVE). However, bacteria present a good growth on MV+Fe indicating that this strain does not present an aminacid auxotrophy.

2- Methionine addition to MVE+ Hemoglobin restore the Sm242 ability to use hemoglobin as iron source. Out of 17 aminoacids and 3 vitamins tested, methionine was the only compound which produce this effect.

3- Methionine addition to the growth medium is not able to restore the growth per se., indicating that it does not act directly on iron acquisition.

4- The phenotype of the iron acquisition mutants tested is the same in Ty rich medium than in MVE+hemoglobin and methionine.

5- The utilization of iron from siderophores and heme-compounds also depends on the presence of methionine in the growth medium

6- Methionine addition to MV produce an increase in the lag phase, suggesting that cells may adapt their metabolism to the presence of this extracellular aminoacid.

7- Cells growth on iron limited medium supplemented with methionine and hemoglobin produce two major outer membrane proteins which are not present in cells growth in iron sufficient medium.

To sum up, we can conclude that hemoglobins and siderophore-mediated iron transport in S.meliloti 242 depends on the presence of methionine in the growth medium. The dependence of aminoacids for iron acquisition has not been previously described and currently its biological significance is still unclear.

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OCCURENCE OF ISATIN HYDROLASE AND PURIFICATION AND CHARACTERIZATION OF THE ENZYME FROM SPECIES OF RHIZOBIA.

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Isatin (indole-2,3-dione) is an intermediate in the degradation of indole-3-acetic acid (IAA) as it occur in strains of *Bradyrhizobium japonicum* (Jensen et al, 1995). The enzyme responsible for conversion of isatin into isatinate, isatin hydrolase, was detected in strains *B. japonicum* as well as in species of other rhizobia such as *Mesorhizobium loti* and *Rhizobium galegae* (Olesen, Jochimsen, 1996). The presence of isatin hydrolase in the latter species represents an enigma, since these species are unable to degrade IAA and unable to metabolize isatinate further into anthranilate.

Cells were grown in 0.04% yeast broth medium at 30°C without shaking and induced by the addition of isatin at 0.2 mol m^{-3} overnight. Extracts prepared from 30 ml and 500 ml cultures, prepared by sonication, were used to determine specific activity upon induction and loaded onto (1x4 cm) Phenyl Sepharose columns for purification, respectively. Elution was performed with a linear gradient in ethylene glycol (0-60%).

Isatin hydrolase from different strains and diverse species such as: *B. japonicum* USDA110, 61A89; *Bradyrhizobium* sp, USDA3399, JA33; *M. loti* NZP2235, R7A; *M. ciceri* Ca7; *M. mediterraneum* Ca36 and *R. galegae* HAMBI 540, HAMBI 1143 exhibited similar Km towards isatin (range $.008\text{-}.014 \text{ mol cm}^{-3}$) and a high affinity towards Phenyl Sepharose and needed ethylene glycol for elution (range 23-38%). This demonstrates that isatin hydrolase is well conserved even among species that have no apparent use of such an activity.

Isatin hydrolase was purified to homogenity from *M. loti* NZP2235 by successive passage onto Butyl Sepharose, Phenyl Sepharose and DEAE Sehadex columns followed by concentration using ultrafiltration. The final preparation revealed one band with a molecular mass of 30.000 upon analysis by SDS PAGE. Elution from a Sephadex G75 gelfiltration column gave a native molecular mass of 31.000, indicating that isatin hydrolase is a monomer. Isatin hydrolase was found to be a metalloenzyme that require mangenese (Mn^{++}) for maximal activity.

Several indole- and anthranilate derivatives were found to induce isatin hydrolase to even higher levels than isatin itself. 4-chloro-anthranilate, anthranilate and IAA were the best inducers in *Bradyrhizobium* sp. ; anthranilate the best within strains of *M. loti*; anthranilate and isatin worked well with *M. ciceri* while 4-indolecarboxylic acid and isatin were the best inducers for *R. alegae*. The presence and induction pattern with indole- and anthranilate compounds of isatin hydrolase in diverse species might indicate that IAA and anthranilate catabolic pathways were commonly present in rhizobia earlier on and elements of such pathways have been lost if not useful to a particular species.

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SYMBIOTIC AND METABOLIC PROPERTIES OF AN α-KETOGLUTARATE DEHYDROGENASE MUTANT OF *BRADYRHIZOBIUM JAPONICUM.*

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We are using an α-ketoglutarate dehydrogenase (KGDH) mutant to investigate the route of dicarboxylic acid catabolism by *B. japonicum* bacteroids. As expected, the mutant cannot grow on acetate but, surprisingly, can grow on malate as its sole carbon source (Green and Emerich, 1997a). Despite having a delayed nodulation phenotype, this mutant eventually forms bacteroids with acetylene reduction activity (Green and Emerich, 1997b).

Using a flow-chamber system to make direct measurements of O_2 consumption and NH_4 excretion (Bergersen and Turner, 1990), we have now confirmed that bacteroids formed by the KGDH mutant display wild-type rates of respiration and nitrogen fixation. Furthermore, the two strains show the same relationship between the rates of respiration and nitrogen fixation, suggesting that they are operating with similar metabolic efficiencies.

Despite the absence of KGDH activity, both whole cells and cell-free extracts of the mutant are able to decarboxylate U-^{14}C α-ketoglutarate at significant rates, indicating that there is some alternative route for α-ketoglutarate catabolism. The activity is CoA-independent, TPP-dependent, and shows non-Michaelis-Menten kinetics with respect to α-ketoglutarate concentration. Since cell-free extracts decarboxylate U-^{14}C glutamate very slowly, the GABA shunt is unlikely to be the pathway responsible for α-ketoglutarate decarboxylation in the mutant.

The CoA-independent α-ketoglutarate decarboxylation activity we have found is similar to an α-ketoglutarate decarboxylase (EC 4.1.1.71) discovered in *Euglena gracilis* mitochondria (Shigeoka et al, 1986). *E. gracilis* mitochondria lack α-ketoglutarate dehydrogenase and instead use α-ketoglutarate decarboxylase and succinate semialdehyde dehydrogenase to catabolize α-ketoglutarate to succinate. *B. japonicum* also has succinate semialdehyde dehydrogenase activity (Kouchi et al, 1991) and, in other rhizobia, this enzyme is required for optimal nitrogen fixation (Fitzmaurice and O'Gara, 1993). We hypothesize that succinate semialdehyde dehydrogenase, along with α-ketoglutarate decarboxylase, participates in an alternative pathway for α-ketoglutarate catabolism, and that this pathway may become important during symbiotic nitrogen fixation. The potential advantage of having the second pathway is that succinate semialdehyde dehydrogenase can reduce NADP instead of NAD, thus avoiding the NADH buildup associated with running the TCA cycle under oxygen-limited conditions. Having both pathways for α-ketoglutarate catabolism would allow *B. japonicum* greater flexibility in partitioning the reductant generated by the TCA cycle in response to changing metabolic demands.

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GENETIC CHARACTERIZATION OF AN INDUCIBLE ASPARAGINASE IN *Rhizobium etli*

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Rhizobium etli can use L-asparagine as the sole carbon and nitrogen source through the action of two enzymes. The first enzyme, asparaginase, catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonium. The second enzyme, L-aspartase, catalyzes the reversible deamination of L-aspartate to yield fumarate and ammonium. *R. etli* has two asparaginases: Asparaginase I migrates faster than asparagine II in native gels stained by activity, it is thermostable, it has a low Km (μM) for asparagine and it is constitutive. Asparaginase II is thermolabile, it has a high Km (mM) towards asparagine and it is induced when asparagine is the nitrogen or carbon source in the culture medium. *R. etli* aspartase also is positively regulated by asparagine. To further characterize the role of the asparaginase II in *R. etli,* we have obtained two mutants with a Tn5-mob transposon, which were unable to grow in asparagine as the sole carbon source. AHZ7 mutant was impaired in their asparaginase II and aspartase activities and AHZ1 was affected only in their aspartase activity. The growths of both mutants in asparagine were restored by complementation with a *R. etli* cosmids bank. We selected a cosmid of 20 kb in length (pAHZ11) which was able to complement both mutants and to restore asparaginase and aspartase activities in AHZ7 mutant, and aspartase activity in AHZ1. The pAHZ11 cosmid was subcloned with EcoRI up to 10.8 kb (pAHZ33), since this subclone was able to complement both mutants, the insert was cloned in pUC18 and sequenced on both strands.

By comparison with reported sequences in GenBank, we found four different open reading frames in the cosmid sequence. The open reading frames were designated *ansR, ansP, orfP* and *asp* and their deduced amino acid sequences showed significant homology to the *Bacillus subtilis* gluconate operon transcriptional represser, the *Salmonella enterica* L-asparagine permease, Orf4 from *Streptomyces coelicolor* and aspartase from different microorganisms, respectively. A number of facts suggests that *ansP, orfP* and *asp* genes are transcribed together, forming an L-asparagine permease operon, which may be controled by ansR: (1) the genes are contiguous and are transcribed in the same direction, (2) exists a short space between the termination codon for *ansP* and the initiation codon for *orfP*, so that between *orfP* and *asp*, (3) upstream from the beginning of *ansP* are present two sequences which are similar to the common -24 and -12 consensus sequence for bacterial promoters σ^{54}-**dependents**, possibly this sequences represents a likely promoter site for the ansP transcript (4) there is not a promotor-like sequence for *orfP* and *asp.*

In native gels stained for L-asparaginase activity, extracts of *R. meliloti* 1021 harboring the cosmid pAHZ33 showed a thermolabile band that migrated as did *R. etli* asparaginase II. This results indicates that one gene present in pAHZ33 presents L-asparaginase activity. It is probable that *orfP* encodes for a protein with L-asparaginase activity. The amino acids forming the putative catalytic triad for L-asparaginases (Thr-89, Asp-90 and Lys-162 in *E. coli* asparaginase II) are localized in positions almost identical near to the carboxil-terminal end in *orfP*.

INDOLE-3-ACETIC ACID PRODUCTION FROM INDOLE-3-ACETONITRILE BY STRAINS OF *BRADYRHIZOBIUM.*

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Microorganisms belonging to the microbial soil communities, particularly the rhizosphere microbiota, have the ability to produce and release an extensive variety of metabolites able to affect plant growth, termed *biologically active substances* (BAS). Many of them, the so called plant growth promoting substances (PGPSs), are considered to be one of the most important factors in soil fertility (W. T. Frankenberger, M. Arshad, 1995).

One of these metabolites, the indole-3-acetic acid (IAA), is a phytohormone involved in several types of microbe-plant interactions. As consequence of its production, some bacteria-plant association become a plant pathogenic process as is the case of *Pseudomonas savastanoi* and olive and oleander plants (N. L. Glass, T. Kosuge, 1988). In others bacteria, as is the case of *Azospirillum,* the IAA production has beneficial effects on the bacteria-plant association acting as a plant growth promotor (Y. Okon, 1985). Rhizobia are symbiotic bacteria having the ability to produce IAA, however, a role for this auxin in the symbiosis has not yet been established, to a great extent as consequence of failed attempts to isolate mutants unable to produce the phytohormone.

Several pathways, usually classified in terms of the more characteristic intermediate, have been reported for the biosynthesis of IAA in bacteria (A. Costacurta, J. Vanderleyden, 1995; C. L. Patten, B. R. Glick, 1996). In *Bradyrhizobium* the indole-3-pyruvic (IPyA) acid pathway was unequivocally described (T. Kaneshiro et al., 1983). and the indole-3-acetamide (IAM) pathway suggested based on the presence of IAM in the culture supenatants and the possession of the indoleacetamide hydrolase (M. Sekine et al., 1988). However neither tryptophan monooxygenase activity nor the monoxygenase gene have ever been found. We have detected two activities: a nitrilase activity capable of converting directly IAN into IAA, and a nitrile hydratase activity that converts IAN into IAM, which can be transformed later into IAA through amidase activity. Nitrile hydratase and amidase activities have been demonstrated in *Agrobacterium, Pseudomonas, Rhodococcus* and also in some *Rhizobium* species (Patten and Glik, 1996). So, it seems that a nitrile hydratase activity is responsible for the production of IAM by bradyrhizobia and not a monooxigenase one.

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POLY-β-HYDROXYBUTYRATE AND GLYCOGEN METABOLISM IN *RHIZOBIUM LEGUMINOSARUM*

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Rhizobium mutants unable to synthesise poly-β-hydroxybutyrate (PHB) enhance nitrogen fixation in certain symbioses such as that of *Rhizobium etli* and bean (Cevallos *et al.*, 1996). Similarly, mutants unable to produce glycogen increase fixation in the *Rhizobium leguminosarum*-bean symbiosis (Downie, personal communication). The effects of these mutations on the pea symbiosis have not been studied. To determine how altering carbon partitioning effects both the pea and bean the symbioses, the PHB & glycogen synthase genes (*phaC & glgA*) of two strains (8401.pRL1JI & 8002) of *Rhizobium leguminosarum* have been mutated. These strains are isogenic, except for differing in Sym plasmid, enabling the effect of the mutations to be compared in pea and bean symbioses respectively. A fragment of *phaC* was amplified from 8002 and an omega cassette (Sp^r) was cloned into a *Psh*AI site. The disrupted fragment was recombined into 8401.pRL1J1 & 8002. The mutants RU1328 (infects pea) & RU1329 (infects bean) were confirmed by Southern blot. PHB was not detected in RU1328 or RU1329. Pea (*Pisum sativum* L. cv. Avola) and bean *(Phaseolus vulgaris* L. cv. Tendergreen) seeds inoculated with the respective WT and PHB mutants were harvested at flowering. Nitrogenase activity of the PHB mutant was 50% less than that of the WT in pea whereas there was no difference in bean (acetylene reduction in μmoles $plant^{-1}hr^{-1}$ were 8401.pRL1JI 5.93 ± 0.59, RU1328 2.99 ± 0.17, $P<0.001$, n = 40; 8002 9.05 ± 0.92, RU1329 10.97 ± 1.11, $P>0.05$, n = 40). There was no difference in biomass accumulation by bean at flowering (46 d) or at pod filling (54 d). There was no difference in pea at flowering (35 d) but plants inoculated with RU1328 were stunted in growth and had a 40% reduction in dry weight at maturity (53 d). As a significant effect was seen in the pea symbiosis free-living RU1328 was characterised physiologically. As PHB is synthesised from acetyl CoA it was considered that preventing PHB synthesis may affect carbon flux through the TCA cycle. To indicate whether a change in flux occurred various TCA enzyme activities were assayed. There was a minor decrease in activity of malate dehydrogenase, citrate synthase and isocitrate dehydrogenase in RU1328 suggesting carbon flux through the TCA cycle was reduced. To confirm this we looked at the release of carbon dioxide from ^{14}C-labelled malate by RU1328 grown on 10mM malate/10mM NH_4Cl. Initial studies show a significant reduction in the rate of CO_2 release from RU1328 indicating flux through the TCA cycle is impaired. Mean doubling times of *R. leguminosarum* strains 8401.pRL1JI & RU1328 grown on 10mM malate/10mM NH_4Cl were 3.46 hrs & 3.24 hrs respectively indicating there is no gross effect in physiology during log phase. Further characterisation of this strain (RU1328) will include measuring reductant levels and O_2 consumption. The experiments will be repeated using Percoll purified bacteroids to determine if metabolism is similarly impaired during symbiosis, and to establish whether the reduction in metabolism can be correlated with decreased nitrogen fixation. Furthermore, mutants in *glgA* (glycogen synthase) have been constructed in 8002 and 8401.pRL1JI to investigate the effect of this mutation in symbiosis. The effect of both *phaC* and *glgA* mutants on partitioning of carbon between PHB and glycogen will be determined by measuring levels of the storage pools in free living bacteria and bacteroids.

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SYNTHESIS AND CATABOLISM OF ALANINE IN *RHIZOBIUM LEGUMINOSARUM.*

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L-Alanine dehydrogenase (AldA) is normally considered to deaminate L-alanine to pyruvate. However, the kinetics of AldA from Rhizobia indicate that under certain physiological conditions AldA may have a role in alanine synthesis rather than in catabolism. This is of particular importance as alanine has been identified as the sole secretion product of nitrogen fixation in *Bradyrhizobium japonicum* (Waters *et al*., 1998. PNAS (USA) 95:12038-12042). In *Rhizobium leguminosarum* both alanine and ammonium are primary secretion products (data this lab). However, the genes involved in alanine synthesis in both free-living bacteria and bacteroids have yet to be determined. The objectives of this work were to identify genes required for alanine synthesis and catabolism and to determine their importance in symbiosis.

Tn*5*-*lacZ* transposon mutagenesis of *R. leguminosarum* bv *viciae* strain 3841 identified a class of mutant, represented by strain RU1275, which did not grow at all on alanine. Sequencing from the Tn*5*-*lacZ* insertion site identified the interrupted gene as a leucine-responsive regulatory protein (Lrp)-type regulator, presumably regulating transcription of genes essential for alanine catabolism.

To identify loci able to complement or suppress RU1275 for growth on alanine a cosmid bank was conjugated into strain RU1275. From the more common class of complementing cosmid sequencing confirmed the presence of the *lrp-type* regulator and also identified two adjacent genes with homology to the D-alanine racemase (*dadX*) and the D-amino acid dehydrogenase (*dadA*). In *E. coli* the *dadAX* operon is essential for catabolism of L-alanine. We propose that in *R. leguminosarum* catabolism of L-alanine normally requires isomerisation to D-alanine and subsequent oxidative deamination by D-amino acid dehydrogenase and transcription of *dadXA* is regulated by *dadR*. The second class of cosmid suppressed the *dadR* mutant. Transposon mutagenesis and screening for loss of suppression identified the interrupted gene as an L-alanine dehydrogenase (*aldA*) with a second *lrp*-type regulator (*aldR*) upstream to and divergent from *aldA*. An *aldA* mutant (RU1327) was constructed by integrating *aldA*::Tn*5*-*lacZ* into strain 3841. Total loss of AldA activity in RU1327 confirmed this to be the only *aldA* present. In symbiosis *aldA* is not essential but bacteroids are defective in alanine secretion.

In summary, we have identified genes important in both alanine synthesis and catabolism. Evidence to support the hypothesis that AldA is primarily responsible for alanine synthesis include; *aldA* is not required for growth with alanine as sole C source, *aldA* was only able to suppress the *dad* mutant when present in multiple copies and *aldA* mutant bacteroids are defective in alanine secretion. Future work includes determining the regulatory effects of the two Lrp-type proteins on transcription of the *aldAR* and *dadAXR* operons and to study further the effects of AldA activity on the pea-*R. leguminosarum* symbiosis.

AMMONIA AND ALANINE EFFLUX FROM NITROGEN-FIXING SOYBEAN BACTEROIDS.

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The bacteroids of soybean root nodules are capable of fixing sufficient N_2 to sustain large crop yields and at the same time contribute significantly to the N-status of the soil in which the plants are grown. For the past thirty years it was assumed that the product of the process was soluble ammonia which was then rapidly assimilated by host pathways into aliphatic amino acids and thence into ureides. More recently, it has been asserted that alanine rather than ammonia is the major end-product of nitrogen fixation in soybean and that the observations described above resulted from alanine deamination by contaminating enzymes in crude bacteroid fractions (Waters et al., 1998). We have reinvestigated this idea using bacteroids isolated anaerobically by differential and gradient centrifugation, in both a closed (shaken) system and an open (flow-through) system (Bergersen and Turner, 1990).

Our results indicate that:
(1) In the flow-through system, when conditions are optimised for maximum rates of respiration and nitrogen fixation, ammonia but not alanine is produced.
(2) When flow chamber conditions are arranged to restrict oxygen supply and allow ammonia to accumulate, small amounts of alanine are sometimes produced. However, respiration and N_2 fixation rates are low and ammonia is still the main product.
(3) In the closed system as described by Waters et al (1998), some alanine is produced but total N_2 fixation rates are very low. Formation of ammonia was not detected under these conditions.
(4) Purification of bacteroids on sucrose or Percoll gradients does not increase alanine production in either system (but it does decrease maximum respiration and N_2 fixation rates).

We conclude that in vivo, when ammonia demand in the plant is high and respiration rates maintained, ammonia will be the major product of the bacteroids. However, when ammonia demand and respiration are restricted, amino acids, including alanine, may be produced from fixed nitrogen

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SECTION VIII

ASSOCIATIVE AND ENDOPHYTIC ASSOCIATIONS

RECENT PROGRESS IN CHARACTERIZATION OF ASSOCIATIVE AND ENDOPHYTIC DIAZOTROPHS AND THEIR INFLUENCE ON HOST PLANT GROWTH

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1. Introduction

The true nitrogen-fixing endosymbionts are exemplified by species of rhizobia whose ability to fix nitrogen as bacteroids within legume cortical cells and to transfer the products to host plants is unequivocable. No defined nitrogen-fixing endophyte or associative organism has yet been definitively proven by Koch's postulates to provide combined N to a plant partner. The associative diazotroph *Azospirillum brasilense,* the best studied species of the *Azospirillum* genus, benefits plant growth probably by providing root-stimulating quantities of indole-3-acetic acid (IAA). The indirect evidence for BNF benefiting plant growth is possibly strongest for *Acetobacter diazotrophicus* and *Herbaspirillum* species which inhabit sugarcane as endophytes in intercellular spaces and xylem. Two papers in this Plenary Session 4 address this issue while the other two are concerned with IAA biosynthesis in *Azospirillum brasilense,* and with various aspects of the interaction between species of *Azoarcus* and rice.

2. Summary of presentations:

It is now well-established that *A. diazotrophicus* benefits sugarcane growth after inoculation of sterile plantlets: Our observation of increased height in plants 30d after inoculation (Sevilla et al. 1988) has been extended to include measurements of mass and N content of roots and shoots 60d post inoculation. The new results presented here (Kennedy et al.) show that wild type *A. diazotrophicus* PA15 was significantly effective in enhancing mass and N content in plants growing without combined N. The Nif^- mutant MAd3A also enhanced plant growth when combined N was present. The inoculated greenhouse plants growing better at 60d retained a growth advantage four months after being transplanted to open field conditions. $^{15}N_2$ uptake occurred in young wildtype inoculated plants, but not in plants inoculated with MAd3A, showing that nitrogen fixation does occur in plants colonized solely by *A. diazotrophicus.* The $^{15}N_2$ incorporated in PA15-colonized plants has not yet been shown to be transferred to plant material. Of significance is that plants always retained the same strain of bacteria with which they were inoculated, and uninoculated plants never acquired PA15 or MAd3A in the greenhouse or field.

In similar types of experiments, Baldani et al. examined sterile sugarcane plantlets inoculated with *A. diazotrophicus* either alone or with species of *Herbaspirillum, Burkholderia,* or *Azospirillum* and subsequently grown for 12 months in 50 kg pots containing unsterilized soil augmented with ^{15}N. In general, plants inoculated with a mixtures of strains were higher in mass and BNF contribution was more significant than in plants inoculated with a single species. In addition, in field experiments two rice cultivars showed variable responses to inoculation with two strains each of *Burkholderia brasilense* and *H. seropedicae.* Increased seed yield was highest and most consistent in both rice cultivars inoculated with *H. seropedicae* strain Z94.

IAA production by *A. brasilense* contributes to the plant growth promotion effect following inoculation. Broek et al reported that indole-3-pyruvate decarboxylase, encoded by *ipdC,* is a key enzyme in complex pathways for IAA biosynthesis. *ipdC* expression is inducible by IAA itself and

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occurs in cultures in early stationary phase, in a manner similar to quorum-sensing in other bacterial systems. *ipdC* expression is also highest at pH5.5. Interestingly, sequence comparison of the *ipdC* promoter with promoter sequences of auxin-inducible plant genes revealed conservation of a particular motif which is the site of repression in plant cells. In order to look for possible IAA-binding proteins in *A. brasilense,* an lAA-binding peptide, D16, was identified within the ABP1 auxin binding protein of maize, and monoclonal antibodies were prepared against D16. Two immunoreacting proteins were identified in extracts of *A. brasilense*; their sizes were 60 kDa and 15 kDa, and they showed similarity to GroEL and GroES, chaperonin proteins found in other bacteria. One of three copies of a *groESL* operon identified by Southern hybridization was sequenced and found to encode products of 60 kDa and 15kDa, corresponding to the immuno-isolated proteins. It is not yet known whether these gene products are IAA binding proteins.

Species of *Azoarcus* were originally isolated from Kallar grass growing in Pakistan by Reinhold-Hurek and colleagues. In this contribution, the group reports that *Azoarcus* species can also be isolated from surface sterilized rice, being more commonly found in wild rice than in cultivated varieties. Also, the diversity of both culturable (isolated as colonies) and non-culturable (examined by PCR using *nifH* primers) diazotrophs was less in wild rice roots than in modern varieties. Phylogenetic analysis suggests that *Azoarcus* should now be split into fouir different genera. With respect to the rice colonization process, it was shown that type IV pili play a role in adhesion and establishment of microcolonies. Pilin subunit gene expression is under the control of a two component regulatory system, and while the gene encoding it is much smaller than those in other bacteria, a pilin IV mutant was nevertheless complemented by a normal length gene from a phytopathogenic species.

3. Comment

Understanding the mechanisms by which endophytic and associative endophytes interact with plants, and especially for the former, how bacteria infect and colonize host plant interiors, is important. Equally important is to establish the relative contribution to plant growth that occurs from BNF in specific endophytes. In addition, it is now clear that other plant growth promoting factors can be produced by endophytes, and the relative contribution of these must also be determined. The use of BNF in sustained production of cereal crops should be a practical goal for our nitrogen fixation research community. Studies of endophytes and associative nitrogen-fixing bacteria are worthy avenues towards reaching that goal.

4. References

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BIOLOGICAL NITROGEN FIXATION (BNF) IN NON-LEGUMINOUS PLANTS: THE ROLE OF ENDOPHYTIC DIAZOTROPHS

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1. Introduction

Much progress has been made in the area of biological nitrogen fixation with non-leguminous plants over the last 10 years. Several new species of nitrogen-fixing bacteria have been identified but special attention has been given to endophytic diazotrophs. The concept of diazotrophic endophytes was introduced to the area of BNF by Döbereiner (1992) although the term endophyte was coined more than a 150 years ago by Leveille (1846) to define a special class of fungi living inside plant tissues. The term was later extended to bacteria by Chanway (1996) who also observed that some bacteria colonize the interior of plant tissues without causing disease symptoms. Although there are several definitions of bacterial endophytes, the role of the endophytic diazotrophs in association with graminaceous plants, is still not yet well understood. Two major crops, sugarcane and rice, have been intensively studied in the last decade because of their potential to associate with endophytic diazotrophs and contribute to nitrogen nutrition of the plant through biological nitrogen fixation. In this paper we present recent advances involving these crops with special emphasis on the response of the plants to inoculation with these endophytic diazotrophic bacteria.

2. Rice

The potential of rice plants to benefit from BNF has been demonstrated several years ago by App et al (1980) using a nitrogen balance carried out on 4 to 6 consecutive crops where contributions of BNF equivalent to 18% of total N accumulated in the plant were detected. Later studies carried by the same group showed that the amount of nitrogen fixed was greatly influenced by the cultivar of rice tested (App et al., 1986). Studies carried out in Brazil with 15 varieties of rice grown in a tank containing ^{15}N-labeled soil also showed differences in the amount of nitrogen fixed, with long-season varieties obtaining more N from BNF than short season varieties (Boddey et al, 1995). Similar results were also demonstrated by Shrestha and Ladha (1996) working with 70 genotypes of rice evaluated in a pot experiment conducted in soil labeled with ^{15}N fertilizer. These authors also observed a significant correlation between the cycle of the variety and amount of nitrogen fixed.

Most of the studies involving BNF quantification did not simultaneously examine the presence of diazotrophic bacteria colonizing these varieties, although several species of nitrogen-fixing bacteria have been isolated from rice roots (Boddey et al, 1995). Endophytic diazotrophic bacteria, such as *Herbaspirillum seropedicae,* are known to colonize rice plants since 1986 when this species first isolated from surface-sterilized roots (Baldani et al, 1986). However, the exploration of its potential has only started recently. Boddey et al (1995) followed the colonizing of different tissues of the variety IR42, considered of high potential to fix nitrogen, by the natural population of *H. seropedicae* and found that its was much higher in the roots as compared to stem and leaves, mainly during the flowering stages when the nitrogenase activity was also maximum as measured by the acetylene reduction technique. A

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new nitrogen- fixing bacterium belonging to the genus *Burkholderia* was isolated in high numbers from rice plants and according to the morphological and physiological characteristics (Baldani, 1996) and 16S rDNA and DNA:DNA homology (Kirchhof, unpublished results) it was provisorily named *Burkholderia brasilensis* (Baldani et al., 1997b). This bacterium use mannitol as the main carbon source and prefers a pH around 4.5. A semi-solid medium called JMV was developed (Baldani, 1996, Baldani et al, submitted) and has allowed its detection in rice as well as cassava, sweet potato and sugarcane plants. A full description of this species will be published elsewhere and it is part of the collaborative work between our group and Dr. A. Hartmann from Germany supported by the BRA-ENV34 project (CNPq/DFG).

While others groups in the world are still debating the importance of nitrogen-fixing endophytes, we have directed our studies to selected strains of *Herbaspirillum seropedicae* and *Burkholderia brasilensis* to inoculate rice plants. This study has so far concentrated on dryland rice which represents about 50% of the Brazilian area cultivated with rice. Two strains of *H. seropedicae* (Z94 and Z67) and two *B. brasilensis* strains (M130 and M209) were selected under gnotobiotic conditions for high BNF contribution to rice var. Guarani as estimated by the ^{15}N isotopic dilution technique (Baldani, 1996; Baldani et al., submitted). A rice inoculation experiment carried out in the greenhouse with these bacteria have shown that they could provide the equivalent to 20% of the total nitrogen incorporated to the plant. The increment on grain yield varied from 19 to 60% depending on the inoculated strains. Field experiments carried during two years (1997 and 1998) with the best strain from both species always showed a significant increment on the grain yield when the plants were inoculated with *H. seropedicae* strain Z94 . An *Azospirillum brasilense* strain (Sp109) was also used but the effect was null or negative. On the other hand, when these strains were inoculated on the variety CNA8305, a better response was observed with the *B. brasilensis* strain M130. It is not clear why the *H. seropedicae* strain responded much better to the variety from which it was selected for (Guarani). It may be possible that an interaction variety/bacteria exists as have been observed by Schloter and Hartman (1998) for two wheat varieties when endophytically colonized by *Azospirillum brasilense* Sp245 and *A. brasilense* Sp7.

3. Sugarcane

The potential of biological nitrogen fixation of sugarcane was demonstrated several years ago by Urquiaga et al (1992) using the ^{15}N isotope dilution technique. The authors showed that over 60% of the plant N was derived from BNF (the equivalent of more than 170 Kg/ha/year) in some Brazilian sugarcane varieties (CB 45-3, SP 70-1143 and Krakatau). This raised the question of which nitrogen-fixing bacterium mainly responsible for the BNF in these varieties. The idea of incorporating the sugarcane juice into the medium at the beginning allowed the identification of a new acid-tolerant bacterium named *Saccharobacter nitrocaptans* (Cavalcante and Döbereiner, 1988) which was latter renamed *Acetobacter diazotrophicus* (Gillis et al., 1989). Using LGIP medium, containing high sugar concentration (10%) and pH 5.5, the ecological distribution of this bacterium was explored. The Most Probable Number (MPN) technique showed that it can occur in high numbers (10^4 to 10^6 cells/g fresh tissue) colonizing endophytically roots, stems and leaves (Reis et al., 1994).
ELISA technique adjusted to evaluate the population of this bacterium in different varieties of sugarcane showed that these numbers are highly underestimated (Silva 1999).

Other nitrogen fixing bacteria also found to colonize sugarcane plants endophytically are species belonging to the genus *Herbaspirillum.* While the species *H. rubrisubalbicans* has scarcely been found in Brazilian sugarcane varieties, the *H. seropedicae* species has been detected in high numbers in many sugarcane varieties including those from Brazil, Australia and India. More recently, the *Burkholderia brasilensis* species isolated from rice plants was also detected colonizing mainly the roots and base of stems of sugarcane. Furthermore , a new species of *Burkholderia,* provisionally called *B. tropicalis,* was also detected colonizing sugarcane plant tissues. The two *Burkholderia* species have many similar characteristics except the 16S rDNA and DNA:DNA homology which is quite different and allowed the creation of new species (Kirchhof, unpublished results). Besides these endophytic diazotrophic bacteria, other nitrogen-fixing bacteria have been frequently isolated from sugarcane such as *A. lipoferum, A. brasilense* and *A. amazonense* (Baldani et al., 1997a) and other not-yet-characterized diazotrophs. Therefore, to define the bacteria responsible for the BNF in sugarcane is not an easy task.

In addition to the large numbers of diazotrophs found associated with sugarcane, it has been observed that abiotic factors affect the dynamic of diazotrophic bacteria in sugarcane plant tissues. High doses of nitrogen were found to inhibit the colonization of sugarcane plants grown in Mexico by the endophyte *A. diazotrophicus* by Fuentes-Ramirez et al. (1999) A reduction in the numbers of *A. diazotrophicus* was also observed in sugarcane grown in India and fertilized with 275 N/ha (Muthukumarasamy et al, 1999). However, in both studies the authors did not find differences in the population of *Herbaspirillum seropedicae* present in the N-fertilized and unfertilized sugarcane plants. A similar study was carried out here at our Center to compare the effect of high dose of nitrogen (300 KgN/ha) on the dynamic of these endophytes naturally colonizing the sugarcane varieties SP 70-1143 and SP 79-2312 (Reis Jr et al, submitted). The authors observed that the population of *A. diazotrophicus* decreased with N fertilization in the variety SP 79-2312 but not in the variety SP70-1143, while the number of *Herbaspirillum* spp. was not affected by N fertilization. The authors also observed that the population of these bacteria were influenced by the time of harvest, becoming reduced at the beginning of the dry season.

Because sugarcane plants propagate by setts (planting material) and therefore almost always carry the original population of diazotrophs in subsequent off-spring, the only feasible possibility to introduce selected strains of endophytic diazotrophs is through the micro-propagation technique. A methodology was developed by Reis et al, (1999) and has been used to evaluate the BNF contribution, mainly of *A. diazotrophicus* strains, to sugarcane. A pot experiment carried out with the variety SP 70-1143 inoculated with strain PAL5 showed an increase in dry matter and weight of stems as compared to the controls (Olivares, unpublished results). Two other exploratory experiments were carried out at COPERSUCAR-SP using a 50L pots containing a poor (Hapludult) and a rich (Haplustox) soil, both fertilized with 3 levels of nitrogen (25, 50 and 100 kg/ha) and inoculated with strain PAL5 (Moraes, 1997). The authors observed an increase due to inoculation in dry matter of these plants in both soils harvested 11 months after planting. However, this effect was much higher when associated with low or medium dose of nitrogen (25 to 50 KgN/ha). The plants showed a dry matter production equivalent to the un-inoculated treatment that was fertilized with double the amount of the nitrogen. A pot experiment carried out in Arizona (USA) using the sugarcane var. SP70-1143 and inoculated with the *A. diazotrophicus* PAL5 and its Nif minus mutant in presence or absence of nitrogen also showed an increase the height of the plants inoculated with that type strains as compared to the Nif minus and controls (Sevilla et al., 1999). However, the authors mentioned that the effect was not always highly significant and suggested that other mechanisms may be involved. Our group has initiated a study to investigate the BNF contribution of the main nitrogen-fixing endophytes to the sugarcane var. SP71-1143. Micro-propagated plants were inoculated individually with A *diazotrophicus* (1), *H. seropedicae* plus *H. rubrisubalbicans* (2), *A. amazonense* plus *Burkholderia* spp (3), and with a combination of treatment 1 and 2, 1 and 3, 2 and 3 and a mixture of all treatments. The experiment was carried out in a 50 kg pots containing a soil labeled with ^{15}N and evaluated 12 months after transplanting to the pots (see Oliveira et al, this symposium). The results showed that BNF contribution was higher when the plants were inoculated with mixtures of strains as compared to the single inoculation. The amount of nitrogen fixed as measured by the ^{15}N isotopic dilution technique of the third leaf of sugarcane plant grown under the pot conditions varied from 5 to 33% of the total N accumulated. It seems that there are an additive or synergystic effect of the *A. diazotrophicus* and *Herbaspirillum* spp with the *A. amazonense* and *Burkholderia* sp used in the inoculant. The data may be underestimating the BNF potential of the sugarcane plant since the micro-propagated plants had a limited space in the pots to develop and were harvested after only 1 year when they are still accumulating sugar. In addition, the plants were also colonized by indigenous nitrogen-fixing bacteria present in the soil which may have provided part of the nitrogen incorporated in the plant even when they were not inoculated.

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ANALYSIS OF GENES FOR NITROGEN FIXATION AND STUDIES OF PLANT GROWTH ENHANCEMENT IN THE DIAZOTROPHIC ENDOPHYTE OF SUGARCANE, *ACETOBACTER DIAZOTROPHICUS*

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1. Introduction

The discovery of the nitrogen-fixing bacterium, *Acetobacter diazotrophicus,* living within sugarcane plants in several world locations (Cavalcante, Dobereiner, 1988; Li, MacRae, 1992; Fuentes-Ramirez et al., 1993; Dong et al., 1995; Bellone et al., 1997; Ureta et al., 1995) has raised a number of questions concerning this bacterium and its association with plants. Which genes, *nif*, *fix*, others unknown, and associated regulatory genes such as *glnD, glnB, glnK* are present in this organism and what role do they play in the biosynthesis and activity of nitrogenase enzyme or effective plant association? How do such bacteria enter, colonize, and persist within their host plant? Does *A. dizotrophicus* provide significant combined N from biological nitrogen fixation to benefit sugarcane growth of sugarcane? Are other plant-growth stimulating factors involved?

2. Identification of a major cluster of *nif/fix* genes in *A. diazotrophicus*

Three overlapping DNA fragments were isolated from *A. diazotrophicus* strain PA15 on the basis of their ability to either complement *nif* mutants of *Azotobacter vinelandii* or to hybridize to the *nifHDK* genes from *Azospirillum brasilense.* All the *nif* genes common to most other diazotrophs and also several "associated" *fix* or *fdx* genes similar to those in subsets of organisms were found in a single cluster of about 32 kb in length (Figure 1). In addition, *modD* and *mcpA* genes were found downstream of *fixABCD*; whether these have an influence on nitrogen fixation or plant colonization has not yet been determined. The overall arrangement of genes in the *nif/fix* cluster of *A. diazotrophicus* is more like that of *A. brasilense* than that of any other diazotroph although the *nifE* through *nifW* region is more like that in *Rhodobacter capsulatus* (Elmerich et al., 1997; Masepohl, Klipp, 1996; Frazzon, Schrank, 1998). In addition, genes encoding a molybdenum transport system, *modABC,* are found associated with a *nif* cluster in both *A. diazotrophicus* and *R. capsulatus.* An additional *mod* gene, *modD,* is separate from *modABC* within the cluster of *A. diazotrophicus,* located downstream of the *fixABCX* genes. Other difference between clusters in *A. diazotrophicus* and *A. brasilense* is that *nifA* and *nifB* of the latter are not linked to other *nif* genes, and *nifQ, nifU* and *fdxB* are absent from the two characterized *A. brasilense nif* clusters. *nifY* is not found in the *A. diazotrophicus* cluster nor is *nifM,* which has so far been identified only in γ group diazotrophs, represented by species of *Klebsiella* and *Azotobacter. A. diazotrophicus* and *A. brasilense* are the only two diazotrophs known to have an *mcpA-like* gene associated with *nif/fix* genes (Scheider, Passaglia, Schrank, personal communication). McpA is involved in chemotaxis in other organisms via its methylation in response to nutrient or other environmental effectors. In a preliminary experiment using 'swarming' plates, an *mcpA*::Tn*5* mutant of *A. diazotrophicus* was apparently unresponsive to sucrose but chemotactic in response to most other carbon sources (S. Lee, unpublished). Because both *A.*

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with monocots, it will be interesting to determine whether McpA is responsible for chemotactic responses to plant exudates for signaling or nutrition in these organisms.

RpoN-modCBA-nifAB-fdxN-ynf1-nifZ-orf12-fixU-nifHDKENX-orf4,1-fdxB-nifQ-orf6-nifUSVW-fixABCX-modD-mcpA

Figure 1. Order of *nif / fix* and other genes in *Acetobacter diazotrophicus*

Isolation of the *nif/fix* cluster of *A. diazotrophicus* has allowed construction of defined Nif$^-$ mutants, one of which has been extremely important in plant growth experiments to test the hypothesis that *A. diazotrophicus* benefits plant growth by providing fixed nitrogen. Mutant MAd3A, carries a *nifD::kan* mutation that prevents nitrogen fixation (Sevilla et al., 1997); its initial use in plant experiments is described in Sevilla et al., 1998; further experiments are described below. Other mutants include *nifA::uidA(gus)* and *nifB::uidA (gus),* used in experiments to analyze factors that influence expression and activity of the NifA transcriptional activator of *nif* gene expression (Teixeira et al., 1999). NifA protein activity is inhibited by O_2, as in all other α group Proteobacterial diazotrophs. While *nifA* gene expression is not influenced by O_2, it is prevented in the presence of ammonium. The mechanism of repression by ammonium appears not to be related to NtrC or the alternative sigma factor σ^N as occurs in several other Proteobacterial diazotrophs (Merrick, Edwards, 1995). Other genes involved in ammonium control and assimilation are currently being characterized in the Meletzus laboratory; these include *glnB, glnK, glnD, glnA, ntrBC,* and *rpoN* (Meletzus et al., 1998; Perlova et al., 1998).

3. Effects on sugarcane growth after inoculation of sterile micropropagated plants

Early evidence for *A. diazotrophicus* contributing fixed N to sugarcane was indirect and included a correlation of this bacterium being present in plants grown in soils with low combined N content (Fuentes-Ramirez et al., 1993; Boddey, 1995), and the finding that atmospheric N_2 is incorporated into sugarcane probably harbouring this organism, indicated by ^{15}N isotope dilution experiments with field or pot-grown plants (Urquiaga et al., 1992). Such plants were likely to carry a mixture of bacteria in unknown varieties and quantities. In the experiments described here, aseptic sugarcane plants of cultivar SP70-1143, a variety known to carry *A. diazotrophicus* and grow vigorously in N-deficient agricultural soils, were micropropagated from meristem tissue and inoculated with the wild type strain *A. diazotrophicus* PA15 or the Nif$^-$ mutant, MAd3A. Four independent experiments have now shown that inoculation with *A. diazotrophicus* alone can enhance the growth of sugarcane under controlled conditions of either limited or excess combined N and that growth enhancement can be sustained in more mature plants. Table 1 shows the results of one experiment in which plants were grown to a 60 d post-inoculation stage; plant biomass and total N were measured. Under N-limiting condition, plants inoculated with wild type strain had significantly higher biomass and total N than plants inoculated with the Nif$^-$ mutant or uninoculated plants. When N was not limiting, inoculation with either the wild type or Nif$^-$ mutant strain resulted in higher plant biomass and total N content when compared to uninoculated plants. Overall, these results are consistent with the possibility, but do not prove, that BNF by *A. diazotrophicus* benefits growth of sugarcane and also indicate, as previously (Sevilla et al., 1998), that there is another growth enhancing factor provided by *A. diazotrophicus.* In addition, wild type inoculated plants transferred to the field 60 days after inoculation and grown another four months had greater cane weight than did uninoculated plants (data not shown). The specific strain used for inoculation persisted in plants at all stages tested while uninoculated plants remained free of *A.*

diazotrophicus even after transfer to the field. No *A. diazotrophicus* cells could be isolated from the sand in greenhouse pots or field soil nor from plant rhizospheres. These observations support the idea that *A. diazotrophicus* is a strict endophyte.

Table 1. Plant dry weight and Total N content of sugarcane plants inoculated with wild type strain PAl5 and *nifD::uidA* mutant strain Mad3A. Measurements were made 60 days post inoculation of plants.

Treatment	Total plant dry weight (g)	Total N content (mg)
(without supplied combined N)		
uninoculated	5.26 d	23.26 d
+ PAl5	8.20 c	46.06 c
+ MAd3A	6.11 cd	30.74 d
(with supplied N, 2 mM ammonium nitrate)		
uninoculated	18.11 b	116.90 b
+ PAl5	28.35 a	160.92 a
+ MAd3A	23.20 a	143.25 a

While the identity of the other factor or factors involved is unknown, the likely candidates are plant growth hormones because auxins, in particular, and also cytokinins, and/or gibberellins sometimes play a role in bacterial-plant interactions. Strains of *A. diazotrophicus* produce high amounts of IAA in culture medium with PA15, the wild-type strain used in the inoculation experiments described here, producing the greatest amount among the several strains tested (Fuentes-Ramirez et al., 1993), and measured in recent experiments to be about 4.8 $\mu g\ ml^{-1}$ (Lee et al, poster presentation, these proceedings). We cannot at this time know what the relative contributions to sugarcane growth enhancement by *A. diazotrophicus* are from BNF and which from the other factor(s). Our isolation of a Tn5 mutant of *A. diazotrophicus* with diminished capacity for IAA biosynthesis and construction of a double IAA^- Nif^- strain will allow key experiments to be carried out.

4. $^{15}N_2$ incorporation in plants inoculated with *A. diazotrophicus* strains

$^{15}N_2$ incorporation experiments with sterile sugarcane plants also gave results consistent with the potential for a role of nitrogen fixation in the *A. diazotrophicus*-sugarcane association. Sugarcane plants inoculated with wild type strain but not with Nif mutants incorporated as much as 0.5% of their total N from nitrogen fixation over a 24 hr period (Sevilla et al, in preparation). Nitrogen fixation was suppressed by combined N. However, we have yet to show that combined ^{15}N from fixed $^{15}N_2$ is incorporated into plant material.

5. Construction and use of *A. diazotrophicus* strains carrying *uidA (gusA)*

The wide host range plasmid pKT230 served as vector for the insertion behind a constitutive promoter of three different genes, each of which could allow *A. diazotrophicus* to be visualized by staining or imaged inside sugarcane. These were *uidA* (Gus), *cobA* (encoding uroporphyrinogen III methyltransferase the product of which fluoresces red), and *gfp* (green fluorescent protein). Of these, only pMS600 expressing Gus was useful. No fluorescence was detected in *A. diazotrophicus* strains carrying either of the other two plasmids although fluorescence was evident in an *Escherichia coli* strain harboring them. Examination of sugarcane roots stained with X-Gluc two days after inoculation with PA15 (pMS600) revealed intense blue staining on the tear wounds created by separating the plantlets before inoculation and also at points of lateral root emergence. After 8 days, blue color was also observed in other parts of stained intact plants including areas immediately above the root-stem junction. At 14 days, bacteria were found in leaves and microscopic examination of fixed cross-sections of X-Gluc and basic fuchsin stained leaves showed blue most intensely in intercellular spaces (data not shown).

6. Tests for excretion of ammonium or amino acids in free-living *A. diazotrophicus* Pal5 cultures

If *A. diazotrophicus* can transfer fixed N to sugarcane plants and or to yeast when the two organisms are cocultivated, as has been shown (Cohjo et al., 1993), then it might be expected that release of ammonium or some other form of fixed N into growth medium would occur in pure liquid cultures. Data from experiments where *A. diazotrophicus* PA15 was grown in LGI medium and monitored for OD, pH, nitrogenase activity and ammonium concentration in the culture supernatant are shown in Table 2. The maximum amount of ammonium found in culture supernatants was **28 μM,** occuring when cells were in stationary phase and nitrogenase activity was no longer detectable. This amount is very low, and not expected to be significant for providing a sufficient source of N for growth of other organisms. In addition, no significant levels of any amino acid were detected in culture supernatants. The question arises whether ammonia(um) is excreted from *A. diazotrophicus* during nitrogen fixation, then is taken up by nearby organisms by diffusion or transport, and results in a lower environmental concentration of fixed N which further enhances nitrogen fixation in *A. diazotrophicus.*

Table 2. Ammonium excreted by cells in liquid cultures of *A. diazotrophicus*

Time (h)	OD_{600}	pH	Nitrogenase activity (nmol C_2H_2/min/OD)	Ammonium (μM)
24	0.64	2.8	0.59	<1
48	0.70	3.6	0.92	2
72	0.85	4.1	<0.01	28
96	0.83	4.1	<0.01	27

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INTERACTIONS OF DIAZOTROPHIC *AZOARCUS* SPP. WITH RICE

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1. Introduction

The root system of plants offers different microhabitats for bacterial growth. In addition to the rhizosphere soil and the root surface, the root interior may be colonized as well: endophytic bacteria have been desribed to spend most of their life cycle inside the plant tissues of healthy plants without causing symptoms of plant damage (Reinhold-Hurek, Hurek, 1998). Several gramineous plants have been described to be associated with endophytic, nitrogen-fixing bacteria such as *Acetobacter diazotrophicus, Herbaspirillum seropedicae* (James, Olivares, 1998; James et al, 1994) or *Azoarcus* sp. (Hurek et al., 1994). Several members of the latter genus have been isolated from the interior of roots and culms of Kallar grass, a pioneer plant on salt-affected, often flooded, low-fertility soils in the Punjab of Pakistan. Their main entry points into roots are near the root tips at the zone of elongation and differentiation, and at the emergence points of lateral roots (Hurek et al., 1994). The cortex is colonized inter- and intracellularly, and bacteria may even penetrate into the stele and xylem vessels. These infection patterns have been detected in Kallar grass, but also in monoaxenically grown rice seedlings. This raised the question whether *Azoarcus* spp. is also naturally associated with rice.

2. Preferential Occurrence of *Azoarcus* spp. in Wild Rice Species and Land Races *of Oryza sativa* in Comparison with Modern Races

Asian wild rice species and cultivated *O. sativa* races were screened for occurrence of endophytes. Our studies indicated that the natural host range of *Azoarcus* spp. extends to rice (Engelhard, Hurek, Reinhold-Hurek, in revision). Populations of culturable diazotrophs in surface-sterilized roots were analyzed by culturing different morphotypes from different dilutions of root macerates. The isolates were characterized by 16S rDNA sequence analysis, and *Azoarcus* species were identified by genomic fingerprints. *A. indigens* and *Azoarcus* sp. group C were detected only rarely, whereas *Azoarcus* sp. group D was frequently found in sampes of flooded plants: in 75% of wild rice from Nepal and Philippines, 66% of land races of *O. sativa* of Nepal, and 60% of modern cultivars from Nepal and Italy. The endophytic populations differed with the rice genotype. *Azoarcus* sp. group D were the predominant putative endophytes in the wild rice species *O. officinalis* (from Nepal) and *O. minuta* (from Philippines). Their numbers were significantly lower in modern rice lines, where numbers of other diazotrophs such as *Azospirillum* spp., *Klebsiella* sp., *Sphingomonas paucimobilis, Burkholderia* sp. and *Azorhizobium caulinodans* were increased. Overall, the diversity of cultured diazotrophs, i.e. the different types of endophytic diazotrophs found per plant, was significantly lower in wild rice species than in modern cultivars. Land races of cultivated rice took an intermediate position. This indicates that in wild rice, interactions with diazotrophs in their root interior might be more specialized.

In order to extend this study by a cultivation-independent approach, we selected two root samples which had been used for isolation, one modern cultivar and one wild rice species. Total DNA

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extracted from the roots was subjected to PCR reactions with nitrogenase gene (*nifH*)-specific primers. Amplification products were cloned, and 16 randomly picked clones from each clone library sequenced. The phylogenetic analysis of these partial nitrogenase gene sequences supported our previous observation that the diversity of diazotrophs was lower in wild rice than in modern cultivars: only 7 different lineages were detected, whereas the sequences in modern rice were spread into 13 lineages. Interestingly, new, deeply branching NifH sequences were detected from both samples, which did not match any known sequences from known, cultured diazotrophs. They were widely distributed among the *Bacteria*, belonging to the alpha and gamma subgroup of the *Proteobacteria* as well as to the Gram-positives, moreover a deeply branching cluster was located in the clade of *anf* (iron only) nitrogenases. Also within the *Azoarcus* spp. NifH cluster, novel sequences were detected. This demonstrates that the diversity of diazotrophs in association with roots is widely underestimated. There are a high number of strains (even species or new genera) which have up to now escaped our cultivation approaches. These up to now uncultured bacteria may play an important role in their ecosystem, however to study their role they need to be cultivated in future.

3. Type IV Pili are Involved in Plant-Microbe Interactions

The first crucial event in the infection process of many animal and human pathogenic bacteria is the attachment to the epithelium of the host. Type IV pili play an essential role in mediating bacterial adherence to and colonization of host cell surfaces. They are virulence factors e.g. in *Neisseria gonorrhoeae, Pseudomonas aeruginosa* and *Moraxella bovis* (Strom, Lory, 1993). Type IV pili are filamenteous cell appendages of 6 nm thickness which are assembled primarily from a single protein subunit, pilin, which consists of approximately 150 amino acid residues. A short, positively charged leader peptide which is cleaved off the prepilin, and a highly conserved N-terminal region are characteristic of type IV pilins (Strom, Lory, 1993).

In *Azoarcus* sp. BH72, we detected a gene *(pilA)* sharing 100% identity with the highly conserved deduced N-terminus of type IV pilins. *PilA* encodes an unusually short (6.4 kDa) protein and is transcribed in an operon with a second gene, *pilB,* which encodes for a 14.2 kDa protein showing weak similarity to FimF, a minor component of type I fimbriae of *Escherichia coli.* Immunofluorescence studies showed that this protein occurs outside the cell and might therefore be a component of the pilus itself. Moreover, mutant analysis showed that both genes are necessary for formation of pili visible in the electron microsope.

Mutant analysis also elucidated the role of these pili in *Azoarcus* sp. BH72. Both genes are involved in the establishment of microcolonies on the root surface of rice seedlings: only 1.1–1.8% of achsils of lateral roots were colonized by mutants in contrast to 29.6% for wild type bacteria or 13.7% for the comlementation mutant. Thus, we demonstrated for the first time that type IV pili play an analogous role in plant-bacteria interactions as in pathogen-mammal relationships (Dörr et al., 1998).

The genomic region upstream of the pilin genes gave hints about the transcriptional regulation of the pilin genes. A sequence highly homologous to the consensus sequence of **$^{54}\sigma$-dependent** promotors was located upstream of *pilA,* as well as sequences similar to NifA and NtrC binding sites. Further upstream, a putative two-component regulatory system was encoded. The genes showed highest similarity to regulators/sensors of virulence factors in phytopathogenic bacteria or of type IV pilin genes in *Pseudomonas aeruginosa.* A marker exchange mutant of the putative regulator indeed drastically reduced transcription of the pilin genes in *Azoarcus* sp. BH72. A transcriptional fusion of the ß-glucuronidase gene to *pilA* allowed analysis of the environmental stimuli for pilin gene transcription in *Azoarcus* sp. BH72. Transcription was constitutive under most conditions tested, however starvation for carbon sources enhanced transcription approximately 4-fold. This environmental signal might indicate to the bacteria that they are in need to colonize a new host and thus enhance transcription of "colonization factors".

4. Role of Multiple P_{II}-Like Proteins in *Azoarcus* sp. BH72

P_{II}-like signal transmitter proteins are known to be involved in the regulation of ammonia assimilation and nitrogen fixation. The presence of two P_{II}-like proteins encoded by *glnB*- and *glnK*-homologues has been described in several species of the *Proteobacteria* (van Heeswick et al., 1996; De Zamaroczy et al., 1996). In *Azoarcus* sp. BH72 we reported for the first time the identification of three functional P_{II}-like proteins (Martin, Hurek, Reinhold-Hurek, submitted). They are encoded in the *glnKamtB, glnYamtY* and the *glnB* operons, the *amt*-genes sharing homologies with ammonium transporter genes. Phylogenetic analyses revealed that *glnK* and *glnY* represent distinct lineages from which the proteobacterial *glnK* clade descents, indicating that they have originated from *glnB* by two early gene duplication events. Details of covalent modification by uridylylation and the effect of gene inactivation on growth are given in the presentation of Martin, Reinhold-Hurek (this volume). The third paralogue GlnY was found to be unusual with respect to its response to ammonia: it was found to be always present in the uridylylated form, which in other proteins occurs only under nitrogen starvation, and is thus not able to respond by differential modification to the nitrogen status of the cell.

Mutant analysis also revealed that the three proteins are differentially involved in the regulation of nitrogen fixation in *Azoarcus* sp. BH72. Expression of structural nitrogenase genes was measured by transcriptional *nifH::gus* fusions. In the wild type, the ***glnB***$^-$ and the ***glnK***$^-$ mutant strains, nitrogenase gene expression was repressed in the presence of the combined nitrogen sources ammonia and nitrate, indicationg that GlnB as well as GlnK are able to sense the cell's nitrogen status resulting in inhibition of the acivity of the transcriptional activator NifA. However, in the ***glnBK***$^-$ double mutant synthesizing only GlnY, nitrogenase gene expression was not repressed in the presence of nitrate and ammonia. This might be due to GlnY beeing present in the uridylylated form, independent of the nitrogen status of the cell, thus leading to *nif* gene expression.

We also detected differential roles of the Gln proteins in posttranslational regulation of nitrogenase activity. In wild type cells of *Azoarcus* sp. BH72, addition of ammonia to nitrogen-fixing cells result in a fast inactivation of nitroganse activity, correlating with the appearance of a nitrogenase iron protein of a higher molecular weight in Western Blot analysis. This phenomenon is in accordance with the switch-off mechanism by ADP-ribosylation of the iron protein found in phototrophic diazotrophs. The ammonium switch off of nitrogenase activity was found to be dependent on P_{II}-like proteins in *Azoarcus* sp. BH72: the single mutant of *glnK* showed neither the decrease in activity nor the formation of the higher molecular weight Fe-protein. The single mutant of *glnB* differed in that nitrogenase activity-switch off was still supported, whereas the nitrogenase modification was not detected. In the double mutant, also both activities were lost. This indicated that nitrogenase activity switch off was dependent on GlnK, whereas nitrogenase modification depended on both, GlnB and GlnK.

Interestingly, also the putative ammonium transporter encoded by *amtB* was involved in posttranslational regulation of nitrogenase activity. Neither activity switch off nor nitrogenase modification upon ammonia addition was detected in the *amtB* mutant. Whether this is due to ammonium import into the cell or to a sensory function of this membrane protein needs to be analyzed.

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ASSOCIATIVE AND ENDOPHYTIC SYMBIOSIS

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1. Introduction

The well known diazotrophs include bacteria belonging to the genera *Azotobacter, Azospirillum, Azoarcus, Herbaspirillum, Acetobacter, Burkholderia, Klebsiella, Pseudomonas, Enterobacter* and *Rhizobium.* They are able to exert positive effects on plants by producing and secreting plant growth regulators (PGRs) such as auxins, gibberellins and cytokinins and/or by supplying biologically fixed-nitrogen. Consequently, germination, root development, mineral nutrition and water utilization are improved. However, due to the complexity of the rhizosphere environment and the infinite possibilities of plant-soil-microbial interactions, the mechanisms of action suggested have not been fully elucidated. Nevertheless, substantial advances have been made (Okon et al. 1998).

2. Biological Nitrogen Fixation (BNF)

Many agriculturally important grasses are associated with endophytic diazotrophic bacteria. They have been isolated mainly from plants in which significant BNF has been demonstrated, particularly in some Brazilian sugar cane and rice cultivars but also in maize, sorghum, palm trees and coffee plants (James, Olivares 1998). In addition, the presence of endophytes has been reported for kallar grass in water logged salty soils in Pakistan (Reinhold-Hurek, Hurek 1998).

There is a general consensus that the plant genotype is the key for obtaining higher contribution of BNF in grasses. Endophytic bacteria, are obligate or facultative endophytes that do not survive well in native soil. They spread apparently via seeds, vegetative propagation, dead plant material and by insect sap feeders. Sites of bacterial multiplication are regions such as xylem vessels and intercellular spaces (James, Olivares 1998). Inputs by BNF to gramineous plants such as rice and sugar cane can be significant, in the order of 10 to 80 kg N ha^{-1} $year^{-1}$. The N_2-fixing activities are attributed in large part to the presence of endophytic diazotrophs in the different plant systems. However, direct evidence for this hypothesis is still lacking (James, Olivares 1998). Nevertheless, by using reporter genes such as *lac*Z and *gus*A fused to *nif* genes and immunogold labeling of nitrogenase proteins, it has been shown that BNF is expressed inside the plant tissue, but quantification has been difficult (Vande Broek et al. 1999).

3. Plant Growth Regulators (PGRs)

Most beneficial bacteria synthesize IAA via the indole-3-pyruvic acid (IpyA) pathway. Other biosynthetic pathways are the tryptophan side chain pathway, the tryptamine pathway, the indole-3-acetonitrile (IAN) pathway and a tryptophan-independent pathway (Arshad, Frankerberger 1998). The increased number of root hairs and lateral roots (through which an improved uptake of water and nutrients can occur) following inoculation with *Azospirillum* is primarily thought to be caused by the bacterial production of IAA (Dobbelaere et al. 1999).

Genes encoding indole-pyruvate decarboxylase (*ipd*C) have been isolated from *Enterobacter, Erwinia and Azospirillum.* An *ipd*C Tn5-mutant of A. brasilense with 10% residual IAA production possesses a reduced ability to promote root hair proliferation (Dobbelaere et al. 1999).

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4. Molecular Ecology

This decade has seen the introduction and development of numerous molecular methods that have greatly enhanced our ability to track introduced diazotrophs, to evaluate their physiological status and their influence on other microbial components of the rhizosphere. The rRNA molecules (16S rRNA and 23S rRNA) are endowed with characteristics that render them extremely useful for ecological studies: they are found in all organisms, their sequences exhibit stretches of varying conservation level, they are found in high copy number, and the 16S rRNA genes of a large portion of the cultivable bacterial species as well as of many of as yet non-cultivable bacteria have already been sequenced (RDP II database). Hybridization with fluorescence-labeled probes enables single cell-detection and the brightness of the fluorescence signal can give a measure of the physiological status of the cell (Asmus et al. 1995). Inocula of engineered PGPR can also be specifically tracked using reporter genes. Bioluminescent genes provide a higher sensitivity than colorimetric methods. The most promising bioluminescence reporter metabolite is the green fluorescence protein (GFP), the product of the *Aequoria victoria* jellyfish *gfp* gene .

5. Physiology and Molecular Genetics of Diazotrophs

Many work focuses on the physiology of *Azospirillum, Herbaspirillum, Acetobacter* and *Azoarcus,* covering BNF and its control, ammonium transport (Van Dommelen et al. 1998), motility, chemotaxis and aerotaxis, flagellar components (Zhulin et al. 1996), cell aggregation and adhesion to roots (Burdman et al. 1999), poly-β-hydroxybutyrate (PHB) content of cells, resistance to stress, electron transport, pectinase activity in *Azospirillum irakense* and mentioned PGR production.

6. Commercial Inoculants

First-generation (wild type) *Azospirillum* inoculants for maize (Azogreen®) are now commercially available in Europe (Lipha, France). Results from field experiments during 6 consecutive seasons consistently showed a better utilization of N-fertilizer by maize plants . A company in South Africa, Soygro (Pty) Ltd. (Potchefstroom) is producing (1998-1999) *Azospirillum* inoculants for 150,000 ha of maize, 12,000 ha of wheat and experimentally 500 ha of sorghum. In field experiments with maize carried out during 5 years (1994-1999) a consistent increase in yield of 10 to 30% above non-inoculated plants has been observed. Also in Mexico, maize and wheat fields are being inoculated with *Azospirillum* leading to higher yields (J. Caballero-Mellado, personal communication).

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MOLECULAR APPROACHES FOR THE INVESTIGATION OF THE DIVERSITY AND LOCALIZATION OF DIAZOTROPHIC BACTERIA

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1. Introduction

The evaluation of the natural diversity of diazotrophic bacteria associated endophytically with different plants is an important and challenging endeavour. The diversity of diazotrophic bacteria can be assessed rapidly at the species level by whole cell hybridization with phylogenetic oligonucleotide probes and at the strain level by genotypic fingerprinting techniques. The knowledge about the diversity and identity of the bacterial partners in endophytic associations with different plants is a prereqisite for the exploitation of the benefits from a productive association, especially if biological nitrogen fixation is concerned. Supposedly, these types of symbiotic associations are rooting in a specialised interaction of plant and bacterial partners, providing a good support of nutrients and suitable microhabitat conditions for the bacteria and ready flow of fixed nitrogen to the plant. It has been demonstrated for *Azospirillum brasilense,* that endophytic colonization of roots is restricted to certain strains within a species (Schloter & Hartmann, 1998). The immunoenrichment technique, starting with monospecific antisera and the application of isolation techniques using nitrogen-free semisolid media as well as genotypic and phenotypic fingerprinting techniques were used to assess the natural diversity of diazotrophs. This approach also could lead to the description of new species. Finally, the endophytic localization of the isolates had to be proven by specific in situ localization and identification techniques.

2. Material and Methods

As nitrogen free semisolid media, NFb, JNFb, LGI and IMV-media were used, which provide a variety of carbon sources and pH-conditions (Döbereiner, 1994). These media were inoculated with dilutions of macerated root material, which had received intensive washing and surface disinfection. Material from pellicle forming vials were transferred to fresh media and finally purified on appropriate agar plates. Isolated bacteria were characterized with biochemical and molecular methods, using a set of 16S- and 23S-rRNA directed oligonucleotide probes both by whole cell hybridization (Amann et al., 1995; Hartmann et al., 2000) and dot blot hybridization (Kirchhof et al., 1998). The 16S rDNAs of bacterial isolates which could not be identified with the existing set of probes were sequenced and new probes were designed using the PROBE DESIGN tools of the software package ARB (Strunk and Ludwig, 1997). Immunofishing was performed using an optimized protocol (see Schloter et al, this volume). Genetic (ERIC- and InterLINE-PCR) as well as phenotypic fingerprinting (e.g. BIOLOG) were used to characterize the isolates further. New species were identified on the basis of DNA-DNA hybridization, 16S rDNA sequence homology and physiological properties.

3. Results and Discussion

A set of new, 16S rRNA-directed, whole cell-binding oligonucleotide probes were developed for several diazotrophic alpha proteobacteria (all species of *Azospirillum* and *Acetobacter*) as well as beta proteobacteria (*Burkholderia* spp. and *Herbaspirillum* spp.) including the new species *Azospirillum doebereinerae* (cand.), *Herbaspirillum frisingense* (cand.).

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A. doebereinerae was isolated from roots of the high yielding C4-energy plant *Miscanthus sinensis,* grown in Freising, Germany. It is closely related *to A. lipoferum, A. largomobilis,* and *A. brasilense*; it cannot utilize glucose, N-acetylglucosamin and D-ribose, but it utilizes fructose. *A. doebereinerae* does not need biotin for growth. So far known, it is not an endophytic diazotroph. Its optmal growth temperature is 30°C.

Herbaspirillum frisingense was isolated from roots of *Miscanthus* and *Pennisetum purpureum* grown in Germany and Brasil. It can be distinguished from *Herbaspirillum seropedicae* and *rubrisubalbicans* by the use of oligonucleotide probes and by phenotypic tests. *H. frisingense* cannot utilize inositol, L-rhamnose and mesoerythritol but grows on N-acetylglucosamine. After inoculation of *Miscanthus* plantlets with *H. frisingense* isolates Mb11 and GSF30, an endophytic localization of these bacteria in the central cylinder could be demonstrated with monospecific antibodies and immunogold labelling. On the root surface, preferred colonization sites, such as the root tip and emerging side roots, were identified using fluorescence labelled oligonucleotide probes and confocal laser scanning microscopy. Using a mixture of monospecific antisera for *H. seropedicae* and *H. rubrisubalbicans* immunofishing experiments were performed with macerates of sugar cane and *Pennisetum* roots. All isolates from sugar cane were classified as *H. rubrisubalbicans,* although none of them had an identical genetic fingerprint as compared to existing isolates. The bacteria isolated from *Pennisetum* could neither be assigned as *H. rubrisualbicans* nor as *H. seropedicae*, although there was a close relationship. They possibly form a new group of *Herbaspirillum* spp. On the basis of their genetic fingerprints, there was only very limited diversity within the isolates.

4. Conclusions

Investigating the diversity of diazotrophic bacteria associated with plant roots of less well studied plants, isolates of well known and new species could be found. The diversity on the strain level was rather restricted for endophytic bacteria in a given plant, which argues for a specificity of colonization. The mechanism of this specificity is not understood.

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ENDOPHYTIC ESTABLISHMENT OF DIAZOTROPHIC BACTERIA IN SUGAR CANE PLANTS

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1. Introduction

Can bacteria living inside plants play a role other than the induction of a phytopathogenic reaction? This question is starting to be answered by the increasing interest of world scientists in bacterial endophytes. For example, recent studies have described the role of bacterial endophytes in the biocontrol of plant pathogens (Kloepper et al., 1991), disease suppression by induction of localised and systemic plant resistance (Duijff et al.,1996), plant growth promotion by hormone production or increased plant nutrition (Fuentes-Ramirez et al., 1995; Baldani et al., 1995). Furthermore, the endophytic establishment of nitrogen fixing bacteria in roots and aerial parts has been considered as the most likely explanation for the biological nitrogen-fixation (BNF) observed in Brazilian sugar cane varieties. It may also indicate a potential strategy to increase the BNF ability of sugar cane by inoculating with selected strains of diazotrophs. However, before such applications can be made, it is essential to firmly establish the endophytic nature of the bacteria being studied. For example, although high numbers of diazotrophic bacteria are frequently isolated from the roots and aerial parts of surface-sterilised sugar cane plants (up to 10^7 cells per g fresh weight), microscopy studies linked with immunoidentification, reporter genes or "*In Situ*" hybridisation must also be performed in order to localise and identify the bacteria, and hence confirm that they can colonise the inner tissues of the plants (Reinhold-Hurek, Hurek, 1998). Therefore, the present study presents data from various experiments designed to evaluate and describe the processes of infection and colonisation of sugar cane plantlets by the endophytes *Acetobacter diazotrophicus, Herbaspirillum seropedicae* and *Herbaspirillum rubrisubalbicans.*

2. Material and Methods

Micropropagated sugar cane plants (interspecific hybrids of *Saccharum* var. SP 70-1143, CB 45-3, NA 5679, B-4362) were obtained according to Hendre et al. (1993). The assays were carried out in growth chambers and utilised two *H. seropedicae* strains (Z67, HRC54), two *H. rubrisubalbicans* strains (M4, HCC103), two *A. diazotrophicus* strains (PR2, PAL5) and an uninoculated control. Two systems were used to evaluate the plant-bacteria interaction:

(a). Plantlets grown in MS liquid medium were inoculated with 0.1 ml of bacterial suspension containing 10^7 cells ml^{-1}. Surface sterilised samples from roots (5 cm length) and aerial parts (1 g) were taken at 1, 2, 4, 7 and 10 d after inoculation to estimate the number of bacteria in association with the plant according to Döbereiner et al. (1995). In parallel, samples from roots, stems and leaves were prepared for light microscopy (LM), epifluorescent microscopy (EFM), transmission (TEM) and scanning electron microscopy (SEM) according to James et al. (1994). In order to identify the bacteria some selected sections were immunogold labelled for LM and TEM according to James et al. (1994), whilst others were immunofluorescense labelled for EFM according da Silva et al. (1998) using polyclonal antibodies raised against the whole bacteria.

(b). Inoculated plantlets were allowed to interact with the bacteria for 7 d and then transferred to glass pots (3.3 1) containing agar-water solid medium (1 1). The pots were closed at the top with cotton and the plants were fed weekly with 10 ml of 1/10 MS solution without nitrogen. Plants

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were harvested at 1, 3, 6 12 months and prepared for bacterial enumeration and microscopy as described above.

3. Results and Conclusions

At 7 d after inoculation the numbers of bacteria associated with the plants growing in a liquid medium had increased with time and ranged from 10^7 to 10^9 cells g^{-1} fresh weight. Less than one day after inoculation both *A. diazotrophicus* and *Herbaspirillum* spp. were seen randomly colonising the entire root surface, predominantly by apolar attachment in monolayers or, less frequently, in clumps. In contrast to the *A. diazotrophicus* strains, the *Herbaspirillum* spp. were only attached to root hairs and to the root cap at a low frequency. Microscopy observations suggested that all three species penetrated the roots mainly via lateral root junctions, but also via epidermal wounds. In addition to these modes of infection, *Acetobacter diazotrophicus* (but not the *Herbaspirillum* spp.) also appeared to infect the root cap, and the *H. rubrisubalbicans* strains entered the leaves via stomata. After infection, there was obvious bacterial multiplication within the root cortex that subsequently spread throughout the intercellular air spaces. It is considered that the principal locations of both *Acetobacter* and *Herbaspirillum* spp. within the roots of Gramineae are the intercellular-spaces, the cell walls, and the lumen of the xylem (James, Olivares, 1998). With regard to the latter location, in order to access the root xylem, the bacteria invaded sites of emerging lateral roots with the endodermal barrier temporarily broken. The bacteria subsequently colonised the intercellular spaces and vascular parenchyma cells adjacent to the xylem, presumably been excreted with the cells debris to the lumen of the xylem by a phenomenon similar to tylosis via pit membranes. Sections of stems showing all three species colonising xylem vessels suggested the systemic translocation of the bacteria via transpiration streams. In addition, leaves of plants inoculated with *H. rubrisubalbicans* showed the bacteria freely multiplying in the apoplast of the mesophyll cells. The persistent endophytic establishment of *A. diazotrophicus* and *Herbaspirillum* spp. in sugar cane has been confirmed in long term experiments (up to 1 year after inoculation), with numbers in the plants ranging from 10^4 to 10^7 cells g^{-1} fresh weight at harvest. Microscopy showed the bacteria in similar locations to those observed within the short-term experiments. These especially included the intercellular and flow apoplast, in which the bacteria are most likely to be physiologically active.

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INVOLVEMENT OF EXTRACELLULAR COMPONENTS IN THE AGGREGATION OF *AZOSPIRILLUM BRASILENSE*

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1. Introduction

The free-living N_2-fixing rhizobacteria of the genus Azospirillum live in close association with plant roots, where it exerts beneficial effects on plant growth and yield of many crops of agronomic importance (Okon, Labandera-Gonzales 1994). Azospirilla are known for their capacity to aggregate and flocculate, and this property may positively affect their dispersal and survival in soil (Sadasivan, Neyra 1985). A better understanding of the aggregation process and the investigation on the advantages that this phenomenon confers to the bacteria under field conditions may enable the development of more efficient and reliable inoculants for agriculture.

2. Results and Discussion

A medium for consistent induction of aggregation of A. brasilense cells was developed and used to study the effects of chemical and physical factors as well as extracellular components involved in this phenomenon. Growth of A. brasilense strain Cd in a high C:N medium using fructose and ammonium chloride as C- and N- sources respectively, resulted in flocculation already visible to the naked eye after 24 h, whereas no cell aggregates were formed after 72 h of growth in low C:N medium (Burdman et al. 1998).

The concentration of exopolysaccharides (EPS) produced by four different strains of A. brasilense differing in their capacity to aggregate increased with the extent of aggregation (Burdman et al. 1998). HPLC analysis revealed that the relative amount of arabinose present in EPS of the strains strongly correlated with the extent of aggregation. A similar picture was observed for the capsular polysaccharide (CPS). Sp72002, a pleiotropic Tn5 mutant impaired in aggregation (Pereg-Gerk et al. 1998), did not show any arabinose in its extracellular polysaccharides. No arabinose was detected in extracellular polysaccharides of bacteria grown in non-inducing aggregation conditions, except to a relatively small amount in the CPS of the super-aggregative mutant strain FAJ0204, kindly supplied by J. Vanderleyden (S. Burdman, unpublished).

In general, no significant differences were observed when comparing protein profiles (SDS-PAGE) of extracellular fractions obtained from the different strains. A clear difference was found in the pattern of the polar flagellin when comparing between aggregating and non-aggregating cultures (Burdman et al. 1998). The polar flagellin was previously proposed to be involved in adhesion processes (Croes et al. 1993). However, the super-aggregating mutant strain (FAJ0204) lacks the polar flagellum (S. Moens, personal communication).

An effective method for temporarily disrupting floes by sonication of the cells for a relatively short period (20 sec) was described (Burdman et al. 1998). Based on this, a suitable bioassay was developed to investigate the nature of biological factors involved in cell-to-cell adhesion leading to aggregation. Essentially, by adding 20 sec-sonicates of high C:N-grown (aggregating) cells (H-cells) to low C:N-grown (non- aggregating) cells (L-cells), the last are able to aggregate and flocculate after 2-3 h. Boiled or proteinase K-treated sonicates originating from H-cells had

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lower aggregation-inducing capacity thus suggesting the involvement of proteins in aggregation (Burdman et al. 1999).

After fractionation of the crude sonicate, both the outer membrane proteins' (OMPs) and the total membrane (mostly OMPs) fractions possessed relatively high aggregation specific activities. The aggregation-inducing capacity of the OMPs' fraction strongly correlated with its protein concentration in the bioassay. Treatment of this fraction with proteinase K also decreased its aggregation-inducing activity (Burdman et al. 1999). When the bioassay was carried out in the presence of different sugars, only arabinose and galactose were able to strongly inhibit aggregation (S. Burdman, unpublished).

These findings suggest that (an) OMP(s) is (are) involved in the aggregation of A. brasilense, and provides new evidence indicating the involvement of extracellular polysaccharides in this phenomenon. Since no qualitative and quantitative differences were found among the different studied strains in their OMPs' profile, it seems that the OMP element plays a constitutive role in aggregation, with the extent of aggregation being determined by the amount and conformation of extracellular polysaccharides

In the last decade, OMPs (and more concretely porins) have been proposed to play a role as adhesins in invasion and adhesion processes in various gram-negative bacteria (De Mot, Vanderleyden 1991, Achouak et al. 1995). It was shown that the 40 to 42 kDa-major outer membrane protein (MOMP) of A. brasilense constitutes about 60% of the total OMPs in this bacterium (Bachhawat, Ghosh 1987). Based on this background, we started to investigate the possibility that the MOMP of A. brasilense can function as an adhesin. This protein was isolated by gel filtration (Superdex-200, FPLC) from A. brasilense strain Cd. Using an antiserum specifically obtained against the purified MOMP, this protein was shown to possess high affinity to roots of corn seedlings in an in vitro adhesion assay. An adhesion screening of OMPs to root extracts of various plant species showed different intensities of affinity for the MOMP with a stronger attachment to root extracts of cereals (sorghum, corn and wheat) in comparison to other plant species (S. Burdman, unpublished).

Degenerate oligonucleotide primers were constructed on the basis of partial internal amino acid sequences of the MOMP, and PCR products were obtained. One of these, a 766-bp fragment, was DIG-labelled and used in Southern hybridization against total DNA and a genomic library of A. brasilense. An E. coli clone containing a 20-kb EcoRI insert in pLAFR3 was identified. We are currently trying to isolate and characterize the gene encoding the A. brasilense MOMP.

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THE CYTOCHROME *cbb*$_3$ (CYTOCHROME *C*) TERMINAL OXIDASE OF *A. BRASILENSE* SP7

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A.brasileme is a free-living N_2-fixing microorganism living in close association with graminaceous plants. As many aerobic N_2-fixers, *Azospirillum* has to face an O_2-paradox: a minimal O_2-concentration is required to allow sufficient energy production in order to support the energy devouring N_2-fixing process, but O_2-concentrations exceeding 2 kPa inactivate the nitrogenase (Hartmann A, Burris RH 1987). *A. brasilense* apparently has reconciled this paradox in an unique fashion. *A. brasilense* expresses a very efficient energy generating system in conditions compatible with N_2-fixation (Zhulin IB et al 1996). Optimizing its energy production at low O_2-concentrations apparently obviates the need for more elevated O_2-levels to support N_2-fixation. Conceivably, at low O_2-tensions *A. brasilense* must generate ATP by using an extremely efficient O_2-dependent respiratory chain, terminated by an oxidase that can cope with low prevalent substrate concentrations (Bergersen FJ, Turner GL 1980). This study aimed at the characterization of the major terminal oxidase active during microaerobiosis (Marchal K et al 1998).

Spectral analysis revealed the presence of at least two terminal oxidase complexes in membrane extracts from *A. brasilense.* Under microaerobic conditions, the presence of an oxidase, showing spectral properties of cyt*cbb*$_3$-type terminal oxidases seemed prevalent. In aerobically grown cells, spectral evidence was indicative for the presence of an alternative heme *b* containing oxidase. Since spectral analysis revealed the cyt*cbb*$_3$ oxidase as the major oxidase during microaerobiosis, the genes encoding the structural subunits (*cytNOQP*) of this oxidase were identified and characterized. A putative Fnr-consensus sequence was identified in the region upstream of the *cytNOQP* operon. As customarily encountered, downstream of the *cytNOQP* operon, a *cytGHIS* operon was located. Since the genomic organization of the *cytNOQP* cluster seems to be well conserved, an Fnr-homologue was expected to be located in the region upstream of *cytN.* However, the DNA sequence of 1.5-kb upstream of the *A. brasilense cytN* gene did not exhibit sequence homology to any coding sequence present in the database. Moreover, the region encompassing ORF280 of *A. brasilense* (Revers et al. submitted), the homologue of ORF277-like proteins, usually located in the immediate vicinity of the *cytNOQP*-like operons seemed not colinear with the *cytNOQP* operon.

As revealed by sequence analysis, the *A. brasilense* cyt*cbb*$_3$ oxidase is a cyt*c* oxidase belonging to the superfamily of heme-copper oxidases (Garcia-Horsman JA et al 1994). The CytN subunit contains the enzyme active center. Conserved histidine residues involved in binding the high-spin/Cu_B reaction center and the low-spin heme *b* are present. An additional histidine residue, suggested to bind and release protons in *B. japonicum* is conserved at position 260. CytP and CytO are heme-containing proteins for which a role in electron transfer from cytochrome *c* to the active center has been postulated. The small subunit CytQ is less conserved and its role is still elusive. However, based on recent reports in *R. sphaeroides* a role in transducing the redox signal, triggered by the electron flow trough the cyt*cbb*$_3$ oxidase to downstream signal transducers has been proposed (Oh JI, Kaplan S 1999). In contrast to cyt*cbb*$_3$ oxidases encountered in other bacteria but similar to

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the cyt*cbb*$_3$ oxidase of *Azoarcus* and *Helicobacter,* the cyt*cbb*$_3$ oxidase of *A. brasilense* lacks an N-terminal amino acid stretch. This may infer that these amino acids, which have been shown to be cytoplasmically located in *B. japonicum* (Zufferey R et al 1996), are not essential for cytochrome oxidase function.

A cyt*cbb*$_3$ knock-out mutant (*cytN* mutant) was completely devoid of cytP or cytO-like proteins, indicating that *A. brasilense* contains a single copy of the cyt*cbb*$_3$-type terminal oxidase. Comparison of the growth and respiration rate of the *A. brasilense* cyt*cbb*$_3$ knock-out mutant and wild type under different conditions revealed the importance of this oxidase in allowing fast growth under microaerobic NH_4^+-supplemented conditions but no major influence on growth under diazotrophic conditions. This is somehow unanticipated in view of the excessive energy demand of the N_2-fixing process and the importance of the high affinity cyt*cbb*$_3$ terminal oxidase during symbiotic N_2-fixation (Preisig O et al 1993, Schlüter A et al 1997). However, since growth rate of the wild type was significantly lower in the absence of NH_4^+ than in its presence we postulate that free-living N_2-fixation, in contrast to symbiotic N_2-fixation, is limited by a factor other than energy, presumably the low NH_4^+-availability. In both N-limited and N-supplemented conditions, the presence of the second alternative oxidase could partially compensate for the lack of the cyt*cbb*$_3$-type terminal oxidase.

As expected an *A. brasilense cytN-gusA* fusion was maximally expressed under microaerobic conditions (2.2 % DO) in a wild-type background. As customarily encountered for Fnr-dependent type II promoters, the Fnr-box is located approximately -41.5-bp upstream of the putative transcription initiation site (Li B et al. 1998, Wing HJ et al 1995). An upstream region of 237-bp, encompassing the Fnr-box was shown to be sufficient for microaerobic upregulation of the *cytNOQP* operon. Furthermore induction of the *cytN-gusA* fusion in a *R. etli* background was clearly dependent on the presence of the heterologous Fnr-protein. Conclusively, the presence of Fnr-homologue, involved in O_2-dependent regulation of the in *A. brasilense cytNOQP* operon is almost certain.

In contrast to the microaerobic upregulation, observed in the wild type, an *A. brasilense cytN-gusA* fusion was aerobically derepressed in a *cytN* mutant background. In view of these findings a possible role of the cyt*cbb*$_3$ oxidase in redox signaling is discussed.

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DIVERSITY OF ENDOPHYTIC DIAZOTROPHS AND MECHANISM OF ENDOPHYTIC COLONIZATION IN DEEPWATER RICE

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Nitrogen supply is critical for improving the yield of any crop including rice which is one of the most important cereal crops in the world. Although a variety of nitrogen fixing bacteria are associated with rice, the amount of nitrogen fixed by them is low and is inefficiently utilized by the rice plant. Naturally occurring endophytic diazotrophs have an advantage over root associated diazotrophs as they colonize the interior rather than the surface of the plants and hence are better placed to exploit carbon substrates supplied by the plants. They can occupy appropriate locations within the plant where necessary conditions for the expression and activity of nitrogenase exist. In order to reduce the dependence of rice agriculture on chemical nitrogen fertilizers it is considered desirable to identify unique rice genotype-diazotrophic endophyte combinations where diazotrophs can stably persist and fix nitrogen inside rice plant. Rice varieties not bred to efficiently respomd to N-fertilizers such as deepwater traditional, wetland cultivated and wild rice varieties have been proposed to harbor diazotrophic endophytic populations (Barraquio et al, 1997).

Five deep water rice varieties, traditionally grown without the input of chemical nitrogen fertilizer in a large lake in Ballia (U.P.), India, were investigated for the presence of nitrogen fixing endophytes. Analysis of the acetylene reducing activity (ARA) and most probable number (MPN) of the endophytic diazotrophs of the deepwater rice showed that the significant ARA was observed in the submerged portions of the plant reaching its maximum near the bottom but MPN was highest in the aerial parts.

Seeds provide a common route for bacterial transmission from generation to generation. After seed germination the seed borne endophytes infect the interior of the developing plants and translocated to the aerial parts eventually resulting in the colonization of the developing seeds of the next generation (James,Olivares, 1997). In view of this, the macerates of surface sterilized seeds were inoculated in the semisolid N-free malate medium for estimating ARA and MPN of the pellicle forming bacteria. Seeds of each variety showed significant ARA and MPN of 10^5-10^6. After 5 serial sub-cultures in the N-free malate medium the pellicle was streaked on plates for isolating bacterial strains.

Ten isolates from each variety representing all the colony-morphotypes from each variety were selected for phylogenetic analysis by ARDRA (amplified ribosomal DNA restriction analysis). PCR amplification of 16s rDNA resulted in specific amplification of 1.4 kb 16s rDNA band from all the isolates which was digested with restriction endonuclease *Rsa*I and resolved on agarose gel for studying polymorphism of bands. Seeds of each rice variety showed 4-5 ARDRA types. Comparison of all the 50 isolates revealed 5 ARDRA types out of which ARDRA type I, II and III were observed in all the rice varieties whereas ARDRA type IV and V were present in 4 rice varieties. All the five ARDRA types showed significant ARA and IAA producing ability. Using *nif*H specific primers a band of ~ 400 bp was amplified in all the 5 ARDRA types. In order to study the plant polymer hydrolyzing ability of the 5 ARDRA types cellulase and pectinase activities were compared on indicator plates.

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An analysis of the presence of 5 ARDRA types in the surface sterilized seeds with and without husk and the seedlings developing from such seeds was performed in order to find out which ARDRA types present in the seeds are able to invade and spread in the seedlings. Aerial parts of the seedlings of two deepwater rice varieties whose seeds harbored 4 ARDRA types did not reveal any endophyte. Aerial parts of the three other deepwater rice varieties showed presence of endophytes belonging to ARDRA type I and II. The observation that ARDRA type I was present in the seeds of all the five rice varieties and that in 3 varieties this ARDRA type migrated from seeds to the aerial parts of the seedlings suggested that it might be a true endophyte. Comparison of the nucleotide sequence of the PCR-amplified 16s rDNA with the database showed strong similarity to *Pantoea agglomerans*. This identification was also corroborated by BIOLOG method of identification.

ARDRA type I strain, identified as *Pantoea agglomerans,* was tagged with *gus*A by conjugative mobilization of a plasmid construct, m*Tn*5SS*gus*A21, carrying constitutively expressed *gus*A gene (Wilson et al, 1995). Inoculation of the rice seedlings with the tagged strain followed by histochemical staining showed that the tagged strain colonized not only the root hairs, root caps, points of emergence of lateral roots but also the stelar region in the Jaisurya variety. Glucuronidase positive strains were recovered from roots, stem and leaves of the inoculated seedlings. Inoculated seedlings also showed consistently increasing ARA with the increase in the age of the seedlings. Treatment of roots with 2,4-D produced several *para*-nodules which were colonized very extensively by the tagged strain.

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CHARACTERIZATION OF INDOLE-3-ACET1C ACID (IAA) PRODUCED BY THE SUGARCANE ENDOPHYTE, *ACETOBACTER DIAZOTROPHICUS,* IN SUGARCANE GROWTH

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Acetobacter diazotrophicus is a true endophyte of sugarcane and often found in plants grown in agricultural areas where nitrogen fertilizer input is low, rather than in regions with higher fertilizer use (Fuentes-Ramirez L.E. et al 1993). Recent results from the laboratory have shown that a mutant strain of *A. diazotrophicus* unable to fix nitrogen (Nif$^-$) did not stimulate growth of young sugarcane in N-limiting growth conditions compared with a strain able to fix nitrogen (Nif$^+$). However, both strains stimulate plant growth if fixed nitrogen is supplied. These results suggest that *A. diazotrophicus* may have the potential for two beneficial effects on sugarcane: one dependent on nitrogen fixation and the other possibly through production of a plant-promoting growth substance (Sevilla et al 1998). Since *A. diazotrophicus* is known to produce IAA, with particularly high amounts produced by strain PAL5 (used in plant inoculation experiments), we speculate that IAA production is a likely explanation for the plant-growth promotion of sugarcane by both the wild type and mutant strain. Therefore, the specific endophytic association between *Acetobacter diazotrophicus* and sugarcane may represent a beneficial symbiosis with the bacterial partner providing fixed N and/or plant growth promoting factors such as IAA.

Our goal is to determine whether IAA production is important for the ability of *A. diazotrophicus* to stimulate plant growth. *A. diazotrophicus* produces IAA in significant quantities, which may be an additional factor in sugarcane growth. To investigate the role of IAA produced by *A. diazotrophicus* in plant growth stimulation, Tn5 mutagenesis was performed. Six Tn5 mutant strains with reduced, or higher levels of IAA, were isolated and investigated. One of these strains, I36E, was further characterized and double mutant strain (*iaa*$^-$*nif*$^-$) has been constructed in the background of I36E by inserting the Chloromphenicol cassette into *nifD* gene. Four strains: (1) wild-type, (2) *nif*$^-$, (3) *iaa*$^-$, and (4) *nif*$^-$*iaa*$^-$, along with a no inoculum control will be used for plant inoculation experiments.

Reference

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DIVERSITY OF PUTATIVE ENDOPHYTIC BACTERIA ISOLATED FROM WILD AND TRADITIONALY-CULTIVATED RICE

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Endophytic bacteria are ubiquitous in most plant species, residing latently or actively colonizing plant tissues. Some endophytic bacteria have been found to possess several benificial effects on host plants such as plant growth stimulation, nitrogen fixation and induction of resistance against plant pathogens. However , the endophytic bacteria that have not yet been shown any beneficial effect on host plant is crucial to understand their evolutional, ecological and physiological roles in plant-microbe interactions and to explore their traits as well as their potential usage as delivery vehicles of desirable properties for crop production and phytoremediation.

Some varieties of rice are traditionally cultivated in slash-and-burn upland fields in mountainous areas of Southeast Asia. In contrast, wild species of *Oryza* (wild rice) grow in marshlands of the tropics and subtropics. Moreover, most wild rice can survive as perennial plants. Therefore, traditionally cultivated rice in upland fields and wild rice possibly harbor unique endophytic bacteria beneficial for the plant growth and different population of endophytes from those in extensively bred modern varieties of rice cultivated in the presence of fertilizers or agrochemicals. However, little is known about non-diazotrophic bacterial endophytes in rice, particularly the non-rhizosphere bacteria in the aerial parts of rice.

Here we focused on the isolation and characterization of endophytic bacteria from the aerial tissues of wild rice and traditionally cultivated rice. Also, we tried to develop an assay system to survey their functions and persistence in rice tissues. Morphologic and 16S rDNA analyses revealed wide phylogenetic divergence among the isolates. Biochemical and 16S rDNA analyses showed that the most frequently isolated groups were *Methylobacterium* sp. in the alpha-subdivision of *Proteobacteria,* and *Curtobacterium* sp. in the high G+C gram-positive group. We assessed various phenotypic traits likely to be involved in the persistence and functions of the bacteria. In this regard, most isolates excreted pectinase, were motile, and had osmotic resistance to 0.6 M sucrose, whose traits may be involved in endophytic features in rice. About 50% of the isolates showed cellulase activity. A few isolates fixed nitrogen, produced indole-3-acetic acid, and formed capsules; these activities partially correlated with the phylogenetic group.

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MOLECULAR CHARACTERIZATION OF PLANT ASSOCIATED NITROGEN-FIXING BACTERIA

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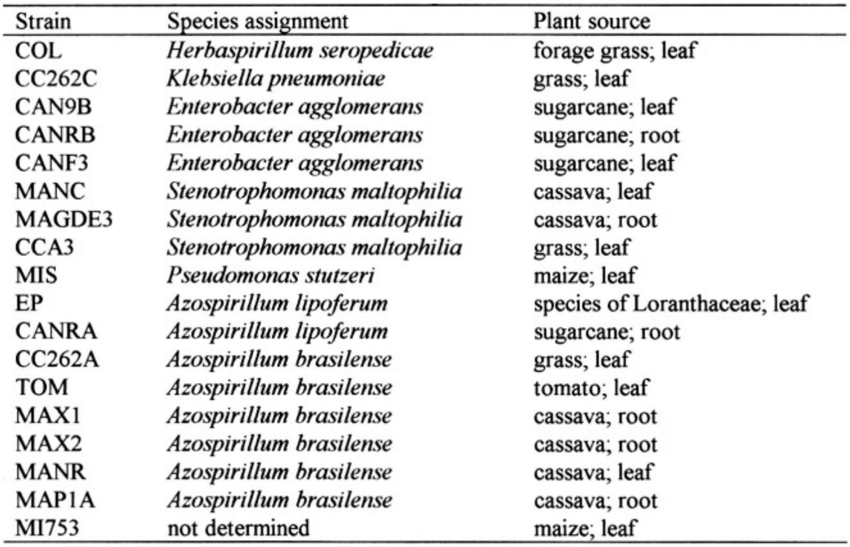
Eighteen strains of nitrogen-fixing bacteria were isolated from leaf and/or root samples obtained from different plant species - maize, sugarcane, tomato, cassava, forage grass and a species of Loranthaceae - collected in rural areas close to the city of São Paulo, SP, Brazil. Bacterial isolates were selected in nitrogen-free media (NFB, Döbereiner et al., 1995). The resulting strains had their nitrogenase activity detected through the ARA test. Strain-specific patterns were obtained by RAPD (Random Amplified Polymorphic DNA) for 10 strains using 5 different randomic primers (OPP-06 and 12, OPC-02, OPA-07 and OPK-20; Operon Technologies, Alameda, CA). The other 8 strains clustered in 4 distinct groups, thus indicating clonal similarities inside each group.

In order to determine phylogenetic relationships among the isolated strains, and between them and other bacterial species, ARDRA (Amplified Ribosomal DNA Restriction Analysis) and 16S rDNA sequence analysis were performed. Relatedness of 17 strains with known species of bacteria was found (Table 1). These results were corroborated by morphological and biochemical tests classically used in bacterial taxonomy. One strain, MI753, could not be assigned to any known species due to inconsistency between phylogenetic and phenotypic data. Dot-blotting experiments using a probe derived from the *nif*H gene (which codes for a nitrogenase subunit) of *Azospirillum brasilense* revealed the presence of this gene in the 3 *S. maltophilia*-like strains and in the strain MI753.



Strain	Species assignment	Plant source
COL	*Herbaspirillum seropedicae*	forage grass; leaf
CC262C	*Klebsiella pneumoniae*	grass; leaf
CAN9B	*Enterobacter agglomerans*	sugarcane; leaf
CANRB	*Enterobacter agglomerans*	sugarcane; root
CANF3	*Enterobacter agglomerans*	sugarcane; leaf
MANC	*Stenotrophomonas maltophilia*	cassava; leaf
MAGDE3	*Stenotrophomonas maltophilia*	cassava; root
CCA3	*Stenotrophomonas maltophilia*	grass; leaf
MIS	*Pseudomonas stutzeri*	maize; leaf
EP	*Azospirillum lipoferum*	species of Loranthaceae; leaf
CANRA	*Azospirillum lipoferum*	sugarcane; root
CC262A	*Azospirillum brasilense*	grass; leaf
TOM	*Azospirillum brasilense*	tomato; leaf
MAX1	*Azospirillum brasilense*	cassava; root
MAX2	*Azospirillum brasilense*	cassava; root
MANR	*Azospirillum brasilense*	cassava; leaf
MAP1A	*Azospirillum brasilense*	cassava; root
MI753	not determined	maize; leaf

Reference
Döbereiner J et al (1995) Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas, Brasília: EMBRAPA-SPI, 60pp.

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SIMULTANEOUS VISUALIZATION OF RHIZOBIA EXPRESSING CFP OR YFP IN PLANT ROOTS

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The green fluorescent protein (GFP) can easily be detected by fluorescence and confocal scanning laser microscopy. Color variants of GFP have been created by genetic engineering. We made use of the GFP variants YFP and CFP (Yellow and Cyan Fluorescent Protein respectively), whose altered excitation and emission spectra (Table 1) allow their separate detection in a single sample.

Table 1. Spectral properties of YFP and CFP

Fluorescent protein	Excitation maximum	Emission maximum
YFP	488	520-550
CFP	457	470-490

Leguminous plants can initiate a symbiosis with bacteria from the family *Rhizobiaceae.* Although this process has been widely studied, many questions still remain. Broad host-range vectors driving expression of YFP and CFP through the LacZ promoter were transferred to the wild-type *Rhizobium leguminosarum* bv. *viciae* strain RBL5560. Mixtures of strains expressing CFP or YFP were inoculated in the growth medium of the leguminous plant *Vicia sativa,* and visualized with a Leica SP confocal laser microscope. Using this method, we were able to visualize the two different bacteria on roots, in infection threads, and in nodules. Initial results indicated that CFP and YFP marked bacteria can be found in the same infection thread (Fig 1). The use of fluorescent bacteria with discernible colors is likely to yield new insights into many processes in which multiple bacterial species are involved, like biofilms, competition, complementation, and of course, the rhizobia-plant symbiosis.

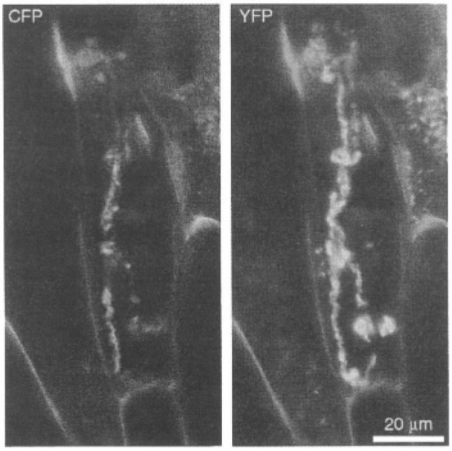

Fig. 1. Visualization of the progeny of at least two different bacteria in a single infection thread. Shown are projections of optical sections acquired with excitation and emission settings specific for CFP or YFP. Both CFP- and YFP- specific images were merged with a simultaneously acquired reflected light image.

BIOLOGICAL NITROGEN FIXATION (BNF) IN MICROPROPAGATED SUGARCANE PLANTS INOCULATED WITH DIFFERENT ENDOPHYTIC DIAZOTROPHIC BACTERIA

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The sugarcane crop in Brazil is known to support the biofuel program called PROALCOOL, however, some problems have affected the expansion of this program, including the need for high inputs of fertilizers and pesticides. Although several endophytic diazotrophic bacteria have been isolated from sugarcane, the role of these endophytic diazotrophs is not yet well understood (Baldani et al., 1997). Previous work has shown that sugarcane variety SP70-1143 is able to obtain up to 60% of total nitrogen from BNF (Urquiaga et al., 1992). On the other hand, responses to inoculation with *Acetobacter diazotrophicus* and *Herbaspirilium* spp. of micro-propagated sugarcane plants have been reported (Sevilla et al., 1998; Oliveira et al., 1995). We used micro-propagated sugarcane var. SP70-1143, to determine the BNF contribution to this crop.
Pots of 60 liter capacity were filled with 50 kg of a non-sterile loamy subsoil and fertilized to avoid any nutrient limitation except N. However, 2.5 grams of ammonium sulfate with 10 atom % ^{15}N excess was added to each pot. In addition, labeled trash was also added to stimulate nitrogen immobilization. The micro-propagated sugarcane plantlets var. SP70-1143, free of bacteria, were obtained from COPERSUCAR (Piracicaba-SP) and inoculated with five endophytic diazotroph species related to sugarcane, according to Reis et al.,(1999). Fifty days after acclimatization, the plants were transferred to the pots. Twelve months after transplanting, the +3 leaf was removed, washed in distilled water, dried and grounded. The ^{15}N isotope excess was determined in a mass spectrometer model Delta Plus (Finnigan). MPN counting was performed to determine colonization in the plant tissues by the inoculated species. Total dry matter accumulation was also evaluated. The inoculation treatment were: *Acetobacter diazotrophicus* (PAL5); *Herbaspirillum seropedicae* (HRC54) + *H. rubrisubalbicans* (HCC103); *Azospirillum amazonense* (CBAmC) + *Burkholderia* sp. (PPe8); treatment 1+2; treatment 1+3; treatment 2+3; treatment 1 +2 + 3; uninoculated control. The MPN counts showed that all bacteria species inoculated could be recovered from the roots in high numbers after 12 months of growth suggesting the establishment of all inoculated diazotrophic endophytes. The non-inoculated plants were also colonized by endophytic diazotrophic bacteria resident in the soil. This may have interfered with the observed amount of nitrogen fixed. The results showed that the nitrogen derived from BNF (%Ndfa) was higher when the plants were inoculated with mixtures of strains compared to inoculation with a single strain and it was equivalent to 33% of the total N. It seems that there are an additive or synergistic effect of the *A. diazotrophicus* and *Herbaspirillum* spp. with the *A. amazonense* and *Burkholderia* sp. used in the inoculant. A tendency to accumulate more dry matter but lower BNF contribution was observed in plants inoculated with *Azospirillum* and *Burkholderia* species, suggesting a possible PGPR effect.

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CHARACTERIZATION OF A SUGARCANE GENE THAT IS INDUCED BY ASSOCIATION WITH ENDOPHYTIC DIAZOTROPHIC BACTERIA

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The association between sugarcane and endophytic diazotrophic was described recently (Dobereiner et al.,1988). Differences on Biological Nitrogen Fixation (BNF) account in distinct sugarcane genotypes suggest that plant genetic factors are controlling the association (Urquiaga et al., 1992). In order to elucidate the role of the plant in the interaction, sugarcane genes induced during the association with *Acetobacter diazotrophicus, Herbaspirillum seropediacae* and *H. rubrisubalbicans* are being isolated using the differential display technique.

MisA2 is one of the isolated genes that are being characterized. It was confirmed by PCR that *misA2* is present in the sugarcane genome. *MisA2* expression analysis revealed that the gene is induced several fold when *in vitro* growing plants are inoculated with the endophytes. Gene expression could not be detected in non-inoculated plants. Induction of expression seems to be general for the association with endophytes and is probably related to bacteria recognition and/or colonization processes, once: (a) it is observed in plants colonized with all the three bacteria species investigated, (b) it could not be detected in plants under various stress conditions (heat-shock, wounding , bacteria infection, fungi infection), (c) it could not be detected in the various organs of plants growing in soil and naturally colonized by endophytes.

The biological function of MisA2 is still unknown. It codifies a putative membrane protein homologous to a yeast protein of unknown function. Although *misA2* plant homologues are not present in the DataBank, we could amplify by PCR DNA fragments homologous to *misA2* in *Arabidopsis thaliana,* tobacco and rice. In order to understand MisA2 function, some tools are currently being used, such as: (a) cellular localization of the *misA2* mRNA and protein, (b) blocking *misA2* expression in sugarcane and heterologous systems (yeast *and Arabidopsis).*

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INTRODUCTION OF THE ATMOSPHERIC NITROGEN FIXING ABILITY TO PLANTS: DIRECT BACTERIUM BOMBARDMENT

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1. Introduction

Particle bombardment is an effective method for delivery of DNA into the intact cells and tissues. The biolistic process has been proven useful in the transformation of numerious and diverse organisms. Currently used biolistic processes involve chemical precipitation of plasmid DNA onto tungsten or gold particles. Only Rasmussen *et al.* (1994) used bacteria as microprojectiles for transformation of plant cells but they used pretreated dead *E. coli* and *Agrobacterium tumefaciens* cells to deliver DNA into plant cell cultures. Living bacteria has not ever been used as microprojectiles. Our concept for creating new type of nitrogen fixing plants is to use the biolistic gun for shooting living *Azotobacter* cells directly into strawberry tissues. Bombardment of living bacteria into the target plant tissues is a very new approach for the biolistic gun.

2. Materials and Methods

Diazotrophic partner was *Azotobacter vinelandii* and *Fragaria* x *ananassa* acted as host plant.
For bacterium bombardment two types of plant inocule were used:

1. Young leaves of micropropagated strawberries, placed onto callus inducing medium 3 days prior to the biolistic treatment
2. Already regenerating callus tisues with 1-3 mm long shoot apexes

5 µl of the microprojectile — consisted of tungsten particles (2-3 µm average size), bacterium cells and spermidin — were shot by 30 bar nitrogen.

3. Results and Discussion

1.) Bombarment of leaves: Three days after the biolistic treatment of the leaves, brownish spots could be observed on the surface which were caused by the tungsten particles. SEM photo clearly shows the penetration sites of the particles. The hole among the epidermal cells is full of the delivered bacteria. Two weeks later yellowish primary calli appeared from the bombarded leaves, probably from the injuries. Examination of these calli by light- and electronmicroscope proves that bacteria were built in the intercellular spaces of the callus tissues.

2.) Bombardment of shoot apexes: To enhance the safety and efficiency of the bacterium delivery and shorten the time after the biolistic treatment, shoot apexes were used as target tissue. The light micrograph of a bombarded meristem shows that bacteria were delivered into the corpus. The electron micrograph demonstrates that the biolistic treatment causes injuries in the internal tissues but both the bacteria and the plant tissue survive the procedure. Continuous division of the meristematic region ensures the spread of the bombarded bacteria in the forming organs and the whole plant.

In both cases plant regeneration was obtained and the delivered nitrogen fixing bacteria could be detected in the intecellular spaces of the regenerated plants.

Bacterium bombardment — first used by us — is a new method for creating artificial type association between diazotrophic bacteria and plants. With this method, theoretically any plant can be inoculated with nitrogen fixing bacteria. Delivery of the bacterium partner is very efficient and safe. As Azotobacters are aerobic organisms they are able to fix nitrogen in all parts of plants even in green, photosynthetically active tissues. From the fact that bacteria can gain access into the newly forming organs and tissues we can conclude that these bacteria can pass through from generation to generation in case of the vegetatively propagated plants.

EFFECT OF INOCULATION OF WHEAT SEEDS WITH *AZOSPIRILLUM BRASILENSE* (245) AND *AZOSPIRILLUM LIPOFERUM* IN FIELD TRIALS.

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Over the last years, several field trials involving seed inoculation of cereals with Azospirillum species have broadly shown contradictory results as to increases in yield. In recent reviews on the effects of field-inoculating such bacteria showed significant results reporting yield increases in about 60 to 70 % of trials (Baldani et al, 1997; Didonet et al., 1996; Bashan, Holguin, 1997). This study aimed to verify the effect of inoculating wheat seeds, cultivar Embrapa 119, under field conditions, on dry matter accumulation, total N, and grain yield.

The wheat seeds were inoculated at seeding time with A. brasilense 245 and isolate 10 of A. lipoferum using peat as a carrier, in addition to an uninoculated treatment. The inoculant having about 10^9 cells of bacteria/g of sterile peat was mixed with the seeds in a proportion of 2.0 g of inoculant for 150 seeds. All treatments (A. brasilense 245, isolate 10 of A. lipoferum, and uninoculated) received 0, 30, and 60 kg N/ha. Biomass accumulation of leaves, stems, spikes, and/or kernels and grain yield were evaluated at anthesis and physiological maturation stages.

Biomass accumulation obtained at anthesis from inoculated plants was significantly higher than the control treatment (Table 1). Such higher accumulation of biomass was probably due to bacterial effect on plant growth, as commonly observed in the initial development stages, resulting in a higher tillering, and consequently spike production. Notwithstanding, such spikes apparently produce less kernels, observed when the number of kernels per unit of area was calculated. Therefore, final grain yield of inoculated plants was not higher. It is pointed out that there was no interaction between N rates and inoculation; only the rates of 30 and 60 kg N/ha showed significant effect.

Table 1. Effect of inoculation of wheat seeds, cv. Embrapa 119, with Azospirillum brasilense 245 and isolate 10 of Azospirillum lipoferum on biomass accumulation at anthesis and grain yield*

	Uninoculated	Strain 245	Isolate 10
Total biomass at anthesis (kg/ha)	4792.0 b	5556.0 a	5431.0 a
Spike biomass at anthesis (kg/ha.	617.0 b	723.0 a	706.0 a
Grain Yield (kg/ha)	3150.0 a	2847.0 a	3214.0 a

*** Significant at 0.05 probability level according to Duncan's test.**

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PHYSIOLOGICAL ASPECTS IN THE METABOLISM OF CARBON AND NITROGEN OF SUGAR CANE (*SACCHARUM* SPP.) COLONIZED WITH ENDOPHYTIC DIAZOTROPHIC BACTERIA.

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In Brazil, sugarcane is grown with relatively low N-fertiliser additions. The use of low N-fertiliser inputs is justified by the results from field experiments (Azeredo et al., 1986), in which significant yield increases due to the application of N-fertiliser were observed in only 19% of the studies. Additionally, recent results (Urquiaga et al 1992) showed that commercial hybrids CB45-3 and SP 70-1143 obtained significant contributions of N from plant associated BNF.
Among the microorganisms isolated from sugarcane plants, *Acetobacter diazotrophicus* (Cavalcante & Dobereiner, 1988) and *Herbaspirillum* sp (Olivares, 1997) were found in high numbers and could explain BNF contributions.

Experiments with sugarcane showed that the number of diazotrophic bacteria change in the plant and was clear that there are preferential sites of colonisation. To try to explain the existence of preferential sites of colonisation, the physiological data of the carbon and nitrogen metabolism were analysed in sugarcane plantlets.

The experiment was conducted in greenhouse conditions. The sugarcane sets were germinated in a tray, with a mixture of sand and vermiculite. Two cultivars were used: SP 70-1143 (high nitrogen fixing genotype) and SP 79-2312 (lower BNF genotype). After 30 days, samples from the leaves and roots were harvested and processed by counting the bacteria number and for physiological analyses.

For the total N, the higher values were found in the shoot of cultivar SP 70-1143 in the set originated from the basal part of the stem, and in the root of cultivar SP 79-2312. No differences were found in relation to nitrate accumulation in the shoot and only a small value was found in root in the set originated from the distal part of the stem. The values of amino-N were higher in the shoot and root of the cultivar SP 79-2312 but showed no correlation with total N. No differences were found in relation to the soluble sugar accumulation, among the origin of the sets and cultivars. As expected, higher levels of soluble sugar were found in the sugarcane shoot. The higher number of *Herbaspirillum seropedicae* and *Acetobacter diazotrophicus* were found in the plants obtained from pieces of basal stems in both varieties. These data corroborate with results obtained from field grown plants. *Herbaspirillum seropedicae* was found in higher number compared with *A. diazotrophicus.* The number of *A. diazotrophicus* was higher in the shoot of SP 70-1143 which showed higher amounts of total N and sugar content. SP 70-1143 was shown in a commercial cultivar to fix higher amounts of nitrogen using ^{15}N data (Urquiaga et al, 1992). The more effective association between the cultivar SP 70-1143 and *A. diazotrophicus,* obtained in this study, corroborates this data.

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PRESENCE OF 16S RRNA GENES IN MULTIPLE REPLICONS IN *AZOSPIRILLUM BRASILENSE*

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Plasmids, ranging in size from 4 to 370 MDa (6- to 550-kb), and megaplasmids (up to 1850 MDa) in *Azospirillum* strains have been described previously. We analyzed 28 *A. brasilense* isolates for plasmid content using the modified Eckhardt method and Southern blots were hybridized with an *E. coli* 16S rDNA probe in order to distinguish chromo-somal and megaplasmid DNA. The *A. brasilense* isolates analyzed harbored from 5 to 7 plasmids (65- to over 1800-kb) and their sizes were in most cases greater than 510-kb. A variety of profiles were observed, but all of the *A. brasilense* strains harbored two very large replicons (1700- and >1800-kb). Most of the strains contained a plasmid with a size of either 570- or 630-kb, and another large 910- or 980-kb plasmid. The 1700-kb replicon and some plasmids around 600-kb strongly hybridized to 16S rDNA genes, while the 910- or 980-kb replicons hy-bridized only slightly. This suggests that the *A. brasilense* genome is composed of multiple minichromosomes instead of a single chromosome. The apparent genome complexity of *A. brasilense* deserves a renewed genetic analysis.

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EFFECTS OF THE INOCULATION OF ENDOPHYTIC DIAZOTROPHIC BACTERIA ON GRAIN YIELD OF TWO RICE VARIETIES (GUARANI AND CNA 8305) GROWN UNDER FIELD CONDITIONS

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Rice plants (*Oryza sativa L.*) are colonized by diazotrophic endophytic bacteria, which can either increase the growth substance production or the biological N2, and therefore contribute to the nitrogen nutrition of the plants. Diazotrophic endophytic bacteria have a large potential of application due to their ability to colonize the plant interior and localise themselves in places protected from oxygen therefore allowing the potential of nitrogen fixation to express at the maximal rate. (Baldani & Döbereiner, 1995). The contribution of biological dinitrogen fixation to rice can be very useful in regions where nitrogen fertilisers are used in very low doses. During the last 25 years rice plants have become very dependent on N fertilisers therefore increasing the production costs (Da Silva et al, 1978). The search for high yields increased N fertiliser use and consequently the cost of production. In addition, ecological problems have resulted in high levels of toxic contents released in the environment. The association of rice roots with endophytic diazotrophic bacteria is being explored envisaging the replacement of N fertilisers by N_2 fixation . Experiments carried out in the greenhouse to select strains of *Herbaspirillum seropedicae* and *Burkholderia brasilensis* were very promising and showed the replacement of N fertilisers by diazotrophic bacteria in rice (Baldani et.al; 1996). To confirm these results, a field experiment was conducted with an area of $1100m^2$. The strains *H. seropedicae* ZAE94 and *Burkholderia brasilensis* Ml 30 were used. Seeds of rice of the varieties Guarani and CNA 8305 (obtained from the Center of Embrapa on Rice and Beans) were pelleted with peat containing 10^8 cell/g of peat, the bacteria were grown in DYGS medium for 24h. The harvest of grain was made 125 days after rice planting. The variety Guarani showed very large increases of grain yields in comparison with the control. An increase of up to 60% was observed with inoculation of ZAE94 of *H. seropedicae* and of 38% with the strain M130 of *Burkholderia brasilensis.* The rice variety CNA 8305 did not show significant differences when inoculated with both, strains. These results show that the inoculation of rice with diazotrophic endophytic bacteria increased yields of rice plants and that the plant genotype affected the association and potential of production in the field. Additional studies are necessary to show whether the effect was due to biological N_2 fixation or other factors associated with the bacteria such as the production of phytohormones.

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N_2-FIXING BACTERIA ASSOCIATED WITH *BRACHIARIA* PASTURES

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Nutritional limitation, including nitrogen depletion, contributes to the degradation process of Brazilian pastures. On the other hand, there is evidence that N-losses could be compensated by biological nitrogen fixation (BNF) associated with some genotypes of *Brachiaria.* These studies indicate that BNF could be responsible for the introduction of 30 to 40 kg of $N\ ha^{-1}\ year^{-1}$ in the plant-soil system (Boddey & Victoria, 1986). Although some pioneer studies were conducted to show the presence of N_2-fixing bacteria associated with *Brachiaria* spp. (Baldani et al., 1981), they were not made over a period of time and neither considered plants under grazing.

The objective of this study was to evaluate the presence of N_2-fixing organisms associated with different genotypes of *Brachiaria* grown under field conditions, under grazing and at different sites.

Diazotrophic bacteria were found in surface-sterilized roots and rhizosphere of the plants, with a number ranging from 10^3 - 10^7. There was no significant difference in the number of these organisms between the three genotypes of *Brachiaria* (*B. decumbens, B. brizantha* and *B. humidicold*) collected at two sites (Goiânia-GO and Uberlândia-MG). The isolation of these organisms showed the predominance of bacteria from the genus *Azospirillum.* At least three types of *Azospirillum* were identified by morphological traits: *A. amazonense, A. brasilense* and *A. lipoferum. A. amazonense*-like organisms were detected at a higher frequency followed by *A. brasilense* and *A. lipoferum* respectively.

All isolates showed the ability to fix nitrogen, but there was a wide range of nitrogenase activity amongst the isolates tested in NFb medium (65 to 250 nmol C_2H_4 mg protein^{-1} h^{-1}) and principally in LGI medium (12 to 205 nmol C_2H_4 mg protein^{-1} h^{-1}).

The use of the ELISA test, utilizing polyclonal antisera raised against these species, grouped these *Brachiaria* isolates and permitted their separation in accordance with the morphological classification.

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DIAZOTROPHIC BACTERIA OCCURRING IN THREE GENOTYPES OF OIL PALM (*ELAEIS GUINEENSIS* JAQUIM.)

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The African oil palm tree is called " Dendê " in Brazil and it originates from west Africa (Gulf of Guiné). The oil produced by this palm is utilised in paints, wax, food, soaps and in lamination of metal. Dendê is also promising for the exploration for a biofuel due to high productivity (4-7 ton/ha/year) and high economic return. Also African oil palm is considered as one of the best options for the occupation of deforested regions of Amazonia as it produces during the whole year, providing year- round employment and has a low impact on the natural ecosystem. In addition, the African oil palm is able to associate with various nitrogen-fixing bacteria which are usually found to colonize roots and stems of these plants (Ferreira et al., 1997 e Shamusdin et al., 1995). The objective of this study was to understand the population of diazotrophs within different ecotypes of the African oil palm and the influence of urea fertiliser on the production of fruit and on the population of natural-occurring **N_2-fixing** bacteria. The experiment was carried out at the "Lemos Maia" Experimental Station CEPEC/CEPLAC (Una-Bahia). Ecotypes utilised were Native Dura Comum and Dura Comum non-Native (original plants, both 8 years old) and Tenera 12 years old. Each sample of plants was divided into leaflets of the leaf 17, rhizosphere soil and roots collected by 10 samples per ecotype for each treatment. The number of diazotrophic bacteria was obtained by the methodology described by Dobereiner et al., (1995), using the most probable number method, applied in two different semi-solid media, without nitrogen. The media used were JNFb (semi-selective for *Herbaspirillum* spp.) and LGI (semi-selective for *A. amazonense*). The experiment was composed of two treatments (addition or not, of nitrogen fertiliser). Each treatment was composed of ten plants. Other nutrients in the mentioned treatments were according to the requirement of the plants. The number of diazotrophic bacteria present in the roots of the native ecotype showed a increase in the summer period (90 days after N application) and a decrease in autumn (180 days) and was not affected by the N application. The Dura ecotype showed a similar behaviour to the native one, even when planted under other soil conditions. The Tenera ecotype showed the opposite, its population decreased in the roots in the summer period and N application had a negative effect on this parameter when JNFB medium was used, even when planted in the same soil type as the Dura ecotype. The ecotype Tenera without nitrogen showed a production of fruit (kg) and also number of fruit branches per plant statistically superior to the other ecotypes. Nitrogen (%) in the roots did not increase after N application in the Tenera ecotype and the productivity was not related to this application. These results showed that this ecotype, which is used to produce oil, had the best performance and did not respond to the addition of N fertiliser .

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INTERACTIONS BETWEEN *AZOARCUS* SP., *GLOMUS INTRARADICES* AND *SORGHUM BICOLOR*

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Most strains of *Azoarcus* spp. were isolated from kallar grass (*Leptochloa fusca* (L.) Kunth). This pioneer plant is grown as forage crop on nutrient-deficient soils in Pakistan without additional fertilizing. Strain BH72 colonizes naturally the interior of kallar grass (Reinhold-Hurek et al., 1993) and also of rice (Hurek et al., 1994), sorghum (Stein et al., 1997) and wheat (Wieland and Fendrik, 1998) when inoculated under laboratory condition. Vesicular-arbuscular mycorrhizal (VAM) fungi form a mutualistic symbiosis with roots of more than 80 % of all plant species. The fungal hyphae penetrate the cortex, develop arbuscles and vesicles inside root cells and thus the common surface between plant and fungus, over which an exchange of carbohydrates and inorganic ions occurs, is extended. The formation of a mycorrhiza depends on nutrient supply, presence of further microorganisms, the way of inoculation and to a great extend on the combination of the symbiotic organisms, since the fungus can not be cultivated without a suitable plant.

The objective of our work was to initiate an association of sorghum (*Sorghum bicolor* (L.) Moench), *Azoarcus* sp. BH72 and *Glomus intraradices.* In order to investigate the reciprocal influence between the three organisms we used a new *in-vitro* cultivation system to transfer the mycorrhiza under controlled conditions which was developed by Dugassa et al. (1995). Already after 3 days hyphae ermerged from a previously mycorrhized leek plant to reach the roots of sorghum and after 3 weeks a symbiosis with intracellular structures was fully established. Root and shoot growth of plants inoculated with either *Azoarcus* sp. BH72 or VAM was enhanced compared to uninoculated control plants. Optimal growth was obtained with both microorganisms.

In the semi-solid medium the formation of a typical pellicle and around some roots a veillike aggregation of bacteria were visible. By microscopic analysis we found co-inoculation with *Azoarcus* sp. BH72 to have a positive influence on the development of the mycorrhiza.

On the other hand the VAM had a positive influence on *Azoarcus* sp. BH72 as well. Nitrogenase activity was higher in cultures together with mycorrhiza (detected by acetylene reduction assay). At any time the activity was significantly higher in mixed culture ($p < 0.05$). Oxygen dependency was tested in pure liquid culture. The bacteria showed maximum activity after 26.5 h with an initial concentration of 5 % O_2. The respiratory metabolism of root and fungus in combination with limited gas diffusion in semi-solid medium created optimal conditions for the microaerophilic *Azoarcus* in the rhizosphere.

The advantage for the plant is obvious: The mycelium can function as an extended root system and therefore helps to scavenge nutrients from the medium. Enhanced nitrogen fixation activity provides more nitrogen, that can be resorbed by the mycorrhiza and finally supports the plant in extending its assimilation parts.

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DOES THE DIAZOTROPH *AZORHIZOBIUM CAULINODANS* IMPART A GROWTH BENEFIT TO WHEAT (*TRITICUM AESTIVUM* L.)?

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Previous studies (Sabry, et al 1997) have shown that, under aseptic conditions, inoculation with *Azorhizobium caulinodans* can increase total dry weight and nitrogen content of wheat plants. The present study aims to determine whether inoculation with *A. caulinodans* benefits the growth of wheat in unsterilised soil, where the bacteria have to survive in the presence of natural soil microflora. The work further attempts to establish whether any effects are due to nitrogen fixation or other growth promoting effects, such as enhanced nutrient uptake as reported by Kapulnik et al (1985) when working with *Azospirillum* sp. To address these aims two experiments were carried out in controlled environments set to simulate late spring conditions in the UK (12h photoperiod and 18/8°C day/night temperatures). Individual wheat plants were grown in pots of sandy loam soil with a low residual N content (10.8 mg N kg^{-1}).

Experiment 1

The growth of wheat plants inoculated with *A. caulinodans* was compared with uninoculated plants. Inoculation was by a seed dressing plus two soil drenches applied at 15 and 45 days after sowing (DAS). Seven destructive harvests were carried out between 28 and 77 DAS and the nitrogen content of shoots was measured at the final harvest. Averaged across all harvests the inoculated plants had a significantly greater total dry weight ($P=0.029$) than the uninoculated plants (inoculated 0.80g; uninoculated 0.56g). The nitrogen content of the shoot was also significantly greater in the inoculated plants ($P=0.017$). From these results it was estimated that inoculation with *A. caulinodans* has a beneficial effect on wheat growth equivalent to the application of 90 kg N ha^{-1}. Experiment 2 was designed to investigate the mechanism(s) responsible for this effect.

Experiment 2

The growth of uninoculated plants was compared with plants inoculated with Nif+ (N fixing) or Nif- (non-N fixing) strains of *A. caulinodans*. Inoculation was carried out by a seed dressing and a single drench at 15 DAS. Averaged across all harvests the inoculated plants had a significantly greater total dry weight ($P=0.023$) than the uninoculated plants (inoculated Nif+ 1.9g; inoculated Nif- 1.8g; uninoculated 1.3g). Over the early harvests the weight of plants inoculated with the Nif+ exceeded those inoculated with the Nif- but the advantage was not maintained. The indications are that the non-nitrogen fixing benefits associated with inoculation are similar in magnitude to those of the albeit transitory nitrogen fixing activity. This leaves two points for further discussion. Firstly, why does the Nif+ establish its advantage so early and secondly, why is this advantage lost mid-way through the experiment?

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PHYSIOLOGICAL EVIDENCE FOR NOVEL TRYPTOPHAN-DEPENDENT PATHWAY FOR INDOLE ACETIC ACID SYNTHESIS IN *AZOSPIRILLUM BRASILENSE.*

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Azospirillum lives in the soil and in association with the roots of grasses and cereals. This bacterium enhances the plant growth primarily by colonization of the root surface that results in increasing the proliferation of the root hairs and of the root system of the host plant. This effect has been tentatively attributed to the production of auxins like compounds such as indole-3-acetic acid (IAA) by the bacterium.

Disruption of the *ipdC* gene in *Azospirillum brasilense* Sp7, a gene involved in IAA production, led to a mutant strain that produced reduced amounts of IAA after growth with a variety of carbon sources when tryptophan or indole was present. A tryptophan auxotroph unable to convert indole to tryptophan, due to a mutation in *trpA* or to *trpB,* produced IÀA when tryptophan was present but did not synthetize IAA from indole. Similar results were obtained when the *trpAB* mutant strain carried an additional mutation in *ipdC* or in *trpD* or both. This suggested the existence of another tryptophan-dependant route for AIA synthesis in *A. brasilense* Sp7.

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REGULATION OF AMMONIUM TRANSPORT IN TOMATO ROOTS COLONIZED BY *AZOSPIRILLUM BRASILENSE*

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The *LEAMT2*-gene, encoding for a high-affinity ammonium transport system in the roots of tomato plants (*Lycopersicon esculentum*) has proven to be strictly controlled by the nitrogen status of the plant. In N-depleted tomato plants the expression of *LEAMT2* becomes a sole function of the externally supplied ammonium concentration provided that the pH of the medium is kept constant. Peak expression is then given in the low micromolar range (2-5μM) of ammonium.

In this study the *LEAMT2*-expression of hydroponically grown plants has been analyzed after the addition of the wildtype strains of *Azoarcus* sp. BH72 and *Azospirillum brasilense* and specific mutants of the latter strain for ten hours (10^7 cfu/ml). These mutants were affected in nitrogenase activity, nitrogen metabolism, or chemotactic properties.

Levels of *LEAMT2*-expression resulting from the use of ^{32}P-radiolabelled *LEAMT2*-cDNA in Northern hybridization experiments were analyzed densitometrically by normalizing individual signals to 18*SrRNA*-derived values and the strongest signal was set to 100%.

With the exception of the nif-minus-mutant, defective in the structural genes of nitrogenase, each strain caused an increase of *LEAMT2*-expression above background level (N-free). Higher levels could be observed in the presence of strains exhibiting a Nif$^+$-phenotype. The strongest signal was detected in the presence of strains C3 and 7028 respectively. Both strains are defective in the *glnA*-gene and therefore constitutively excrete ammonium. Although strain 7028 does not fix nitrogen it excretes detectable levels of ammonium originating from internal sources. Besides the wild type strains strain 7628b also caused a stronger increase. Although defective in the *glnB*-gene strain 7628b is transformed with a truncated but functional *nifA*-gene causing a constitutive Nif$^+$-phenotype. Strain 7628, defective in *glnB* but lacking *nifA* caused a signal of lower intensity. Strain 7611, defective in *glnZ* encoding for an alternative PII-protein showed the highest nitrogenase activity but caused only a low increase of transcript levels. The motility-mutant which shows no tactical response to oxygen or nutritional attractants is a poor root colonizer and caused only a slight increase of *LEAMT2*-expression. This might indicate the importance of a direct contact between associative bacteria and their host plants in order to establish an association with a beneficial effect.

In summary the results suggest that root-associated nitrogen-fixing bacteria do excrete NH_4^+ to levels that can be sensed by tomato roots. This is in agreement with the induction of expression of *LEAMT2* with as low as ≥ 2μM external NH_4^+. *LEAMT2* might be used as a sensitive transcriptional reporter for the ammonium supply of a host plant.

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ENERGY GENERATION VIA EXTRACELLULAR ALDOSE OXIDATION IN CULTURES OF ACETOBACTER DIAZOTROPHICUS FIXING N_2

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A. diazotrophicus is a diazotrophic bacterium that colonizes sugarcane. This organism oxidizes extracellularly glucose to gluconate prior to further sugar metabolism (Stephan *et al.* 1991). A membrane-bound glucose dehydrogenase (GDH) quinoenzyme (containing pyrroloquinoline quinone (PQQ) as the prosthetic group) is involved (Galar, Boiardi 1995). Gluconate is incorporated by *A. diazotrophicus* and further oxidized via the pentose monophosphate pathway and TCA cycle. *A. diazotrophicus* can be grown in chemostat with atmospheric N_2 as the nitrogen source provided that the dissolved oxygen remains below 1.0-2.0 % air saturation. The biomass yields of *A. diazotrophicus* growing under no-fixing conditions are very low compared with other heterotrophic bacteria. As expected the biomass yields under N_2 fixing conditions decreased (compared to the culture with ammonium) using gluconate as the C source but, surprisingly, this yield is not significantly modified using glucose as C source. We conducted chemostat experiments to determine energy generation with different carbon substrates.

By using a model describing growth by a series of equations accounting for the assimilation of the C source into biomass, catabolism, product formation and respiration it is possible to obtain a relationship between growth and product formation with internal metabolic fluxes, which are linked to production and consumption of energy (ATP and reducing power). From such a model we could determine that *A. diazotrophicus,* growing on glucose under N_2 fixing conditions, was able to redirect the electron flow through a more efficient branch of the respiratory chain showing a P/O ratio of 0.7 instead of 0.5 as obtained with gluconate. By using mixtures of xylose and gluconate (as C and energy sources) in chemostat cultures of this organism, the biomass yields were as high as with glucose. Notice that xylose can be oxidized to xylonate via GDH with the concomitant production of reduced PQQ. Xylonate is not further metabolized by *A. diazotrophicus* and is accumulated in the culture supernatants. Therefore, the expression of GDH under N_2 fixing conditions led *A. diazotrophicus* to maximize it efficiency of respiratory energy generation.

For further confirmation we have obtained GDH negative mutants using EMS as mutagenic agent. The mutant screening was carried out in a media containing $CaCO_3$ where the wild type produced a round of solubility and the GDH mutants were unable to do that. Mutants form “pellets ” in cultures with glucose, with either ammonium or N_2. We were able to grow them (avoiding pellet formation) in chemostat cultures with glucose and ammonium. Biomass yields were comparable to those of the wild type under the same conditions. No cultures of these mutants under N_2 fixing conditions were already carried out because it was not possible yet to start chemostat cultures of these organisms in a medium deprived of a source of fixed nitrogen.

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IN SITU LOCALIZATION OF *HERBASPIRILLUM FRISINGENSE* CAND. IN THE ENERGY PLANT *MISCANTHUS*

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Miscanthus is a C_4-energy crop with a very high biomass production (30-50 to. dry material/a). The lignocellulosic biomass crop is utilized for the production of paper pulp or to replace fossil energy. In various field experiments it has been shown that the biomass yield does not increase with the application of mineral nitrogen fertilizer. In other C_4-plants, e.g. sugarcane, maize and sorghum, diazotrophic bacteria, like *Herbaspirillum rubrisubalbicans* or *H. seropedicae*, are suspected to contribute to the biological nitrogen fixation capability of those plants.

Using N-free semisolid media diazotrophic bacteria were isolated from the roots of *Miscanthus* (up to 10^6 /g fresh weight). We identified, among other bacteria (Kirchhof et al 1997), a new *Herbaspirillum* species (*H. frisingense* cand.) and a new *Azospirillum* species (*A. doebereinerae* cand.). To evaluate the colonizing potential of *H. frisingense* cand., axenically grown *Miscanthus* plants were inoculated with two different strains (10^7 cells of strain Mb11 or GSF30 per root). Based on the PROBE DESIGN tool of the sequence analysis software package ARB (Strunk et al 1997) we designed two new 16S rRNA targeting probes for *in situ* hybridization experiments: the probe HERB 68, which is specific for the genus *Herbaspirillum* and the probe Hfris 445, which is specific for the new *Herbaspirillum* species. Whole root pieces and root extract from naturally grown field plants were hybridized with fluorescently labelled oligonucleotide probes and analyzed by confocal Laser-Scanning Microscopy (Amann et al 1996). To investigate the endophytic colonization, thin-sections from inoculated roots were utilized for immunogold labeling (James et al 1994) using polyclonal antibodies (Schloter et al 1997) raised in rabbits reacting with the new *Herbaspirillum* species.

Exploiting the confocal Laser Scanning microscopical images we were able to detect the new *Herbaspirillum* species on the surface of root pieces from inoculated axenically grown *Miscanthus* plants. The bacterial colonization was not even distributed over the root surface. Bacteria occurred mainly on the root tips and at the lateral root junctions. Moreover, by performing a phylogenetic top to bottom approach for the *in situ* identification, cells of *H. frisingense* cand. were found in root extracts of naturally grown *Miscanthus* field plants.

With immunogold labeling it was possible to show that *H. frisingense* cand. Mb11 and GSF30 colonize endophytically xylem vessels, intercellular spaces and epidermical cells of roots from healthy *Miscanthus* plants.

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INTERCROPPING OF COINOCULATED SOYBEAN AND SUGAR CANE PRODUCTIVITY

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The interest in combined inoculation of legume plants with symbiotic nitrogen fixing bacteria and PGPRs (Plant Growth Promoting Rizobacteria) is increasing within the nitrogen fixation field. Recent reports inform about a positive effect on development of legumes produced by the coinoculation of *Rhizobium* and *Bradyrhizobium,* with *Azospirillum*, a diazotrophic PGPR (Burdman et al 1998). Usually *Azospirillum* is associated with *Gramineae* roots, like sugar cane, rice, corn, etc. This association is traduced in a beneficial effect on plant nitrogen nutrition. In Cuban soils, significant increases in productivity of sugar cane have been found when this plant was inoculated with different amounts of *Azospirillum* cultures (Roldós et al 1995). Moreover, *Azospirillum* has been associated with other PGPRs belonging to *Bacillus* genera, obtaining significant increases in N_2ase activity in mixed inocula of *Azospirillum* and *Bacillus* (Khammas et al 1997; Casas et al 998).

The aim of this work was to evaluate the effect of coinoculation of *Azospirillum, Bradyrhizobium* and *Bacillus pumilus* on soybean plants. In parallel, inoculation with *Sinorhizobium fredii* was carried out in order to compare with *Bradyrhizobium* and the possibility to be introduced in Cuban soils. Field experiments in the Matanzas (Cuba) area are being performed to determine the effect of coinoculation of soybean in intercropping with sugar cane *(Saccharum qfficinarum)* on the final yield of sugar, as an ecological alternative to improve sugar cane productivity.

Experiments have been carried out at green house and field level. In the former, seeds of soybean were sown in sterile conditions and inoculated with *B. japonicum* USDA 110, *A. brasilense* 8-INICA and *B. pumilus* B-12. All possible combinations between these strains were performed. The strain SMH-12 of *S. fredii* was also used. Plants were watered with nitrogen free solution with the exception of controls and of the plants inoculated with B-12, because it is a PGPR, but not diazotrophic bacterium. In field experiments, rows of soybean plants inoculated with the above strains were inserted with rows of sugar cane.

The results indicate: i) Bacterial colonization of soybean roots in plants inoculated only with *Azospirillum.* The possibility that this bacterium correspond to the 8-I strain has been analysed by immunolocalization. ii) Any type of coinoculation produces similar or better results than plants watered with nitrate, which indicates that it is not necessarily nitrogen fertilization in coinoculated plants and iii) Cooinoculation of *B. japonicum* USDA 110 with *B. pumilus* B-l is the best combination studied.

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HERBASPIRILLUM FRISINGENSE SP. NOV., A NEW BACTERIAL SPECIES OCCURRING IN C4-ENERGY PLANTS

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The isolation of diazotrophic bacteria using nitrogen-free semi-solid NFb and JNFb media from the C4 fiber plants *Spartina pectinata*, *Miscanthus sinensis*, *M. sacchariflorus* and *Pennisetum purpureum* led to the separation of *Herbaspirillum*-like bacteria (Kirchhof et al 1997). Phylogenetic analysis comparing 16S rDNA sequences of reference strains as well as the new isolates affiliated the new strains within the genus *Herbaspirilium.* By testing the physiological properties, species with different carbon-source utilization patterns were observed (Table).

	H. seropedicae	*H. rubrisubalbicans*	*H. frisingense* sp. nov.	*Herbaspirillum* species 3a	*Herbaspirillum* species 3b
m-erythritol	-	+	-	+	-
L-rhamnose	+	-	-	-	-
m-inositol	+	-	-	-	-
N-acetylglucosamine	+	-	+	+	+
sebacate	-	-	-	+	+

The nitrogen-fixing capability of those isolates was examined by PCR amplification of the *nifD* gene (Stoltzfus et al 1997). All strains were positive for acetylene-reduction activity. Performing DNA:DNA hybridization experiments of chromosomal DNA the newly isolated *Herbaspirillum*-like strains grouped together with similarity values between 63 and 100 %, implying the formation of a new species in the genus *Herbaspirillum,* while hybridization with the other *Herbaspirillum* species resulted in values mostly below 30%. Applying the PROBE DESIGN tool of the sequence analysis software ARB (Strunk and Ludwig 1997) diagnostic oligonucleotide probes based on 16S rDNA specific for the whole genus and for each species could be designed. They were utilized for *in situ* hybridization experiments in the identification of separated strains and the specific localization of herbaspirilla associated with plant tissues (Eckert et al, this volme).

PCR fingerprinting was applied to investigate the relative clonal relationship of a set of isolates (Smida et al 1996). The bacterial strains form an unique band pattern due to their date of isolation and plant origin. These findings further indicate that the level of genetic diversity of bacterial strains may be correlated to their habitat origin (McArthur et al 1988). Moreover, the selective conditions of the endophytic habitat as well as the fiber plant reproduction via rhizomes, which may influence the distribution of the plant associated bacteria, possibly cause the limited genomic variability observed among the *Herbaspirillum frisingense* sp. nov. isolates from C4 fiber plants.

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THE RESPIRATORY SYSTEM AND THE DIAZOTROPHIC ACTIVITY OF *ACETOBACTER DIAZOTROPHICUS.*

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The nature of the respiratory system of *A. diazotrophicus* was investigated. Cells obtained from well aerated cultures possessed a highly active, membrane-bound electron transport system composed of dehydrogenases of NADH, glucose, acetaldehyde, ethanol, succinate and gluconate. Spectral analysis revealed the presence of cytochromes of type *b, c,* and *a.* These were confirmed by HPLC analysis of hemes and stain of five cytochromes c in PAGE-SDS were detected. Their functions as electron carries, located before the ubiquinone pool, is discussed. Cytochrome a_1 seems to be the major oxidase activity during aerobic diazotrophy. Addition of 20mM $(NH_4)_2SO_4$ to cultures caused the nitrogenase repression and expression of Cyt *d.* Nitrogenase activity levels were higher in cells grown in sucrose as carbon source and well aerated cultures, which strongly suggested that a mechanism of respiratory protection of the nitrogenase operates in *A. diazotrophicus* Pal5. In order to begin to understand the essential role as terminal oxidase of Cytochrome a_1 , during aerotolerant nitrogen fixation, the structural genes were cloned by amplification of its genomic DNA by PCR.

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DEVELOPMENT AND APPLICATION OF NEW OLIGONUCLEOTIDE PROBES FOR THE *IN SITU* IDENTIFICATION AND LOCALISATION OF NITROGEN FIXING BACTERIA

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Many diverse nitrogen fixing, plant associated bacteria were isolated all over the world from various non legumes plants. A lot of different species were commonly found in the rhizosphere soil, the rhizoplane or in the interior of the plants. The classification and identification of diazotrophic bacterial isolates with classical microbiological methods is often difficult and time consuming. Therefore we developed new 16S rRNA-targeted oligonucleotide probes for the fluorescence *in situ* hybridization of N_2-fixing bacteria belonging to the alpha- and beta-subclasses of *Proteobacteria.*

For the probe development we used the PROBE DESIGN tool of the software package ARB (Strunk O, Ludwig W, 1997). The new probes are targeted to the 16S rRNA and were labeled with the fluorescence dyes FLUOS, Cy3 and Cy5. The specificity and sensitivity of the probes were evaluated by *in situ* hybridization to mixtures of whole fixed cells of selected reference strains. Stringent hybridization conditions were adjusted by gradually increasing the formamide concentration in the hybridization buffer while keeping the ionic strength (0.9 M sodium chloride) and hybridization temperature (46°C) constant. The preparation, fixation and hybridization of root extracts and root pieces were performed as described by Assmus et al. (1995).

The new probe set for the *in situ* identification of N_2-fixing bacteria includes the genus-cluster probes *Acetobacter-Gluconobacter* (Neef, 1997) and *Burkholderia-Suttorella* (Stoffels et al., 1998), genus-specific probes for the genera *Azospirillum, Sinorhizobium* and *Herbaspirillum* as well as the species cluster probes *B. cepacia-B. vietnamiensis* (Stoffels et al, 1998) and *B. gladiolii-B. glumae-B. vandii-B. plantarii-B. cocovenenans.* In addition we developed species-specific probes for the three *Herbaspirillum* species *H. seropedicae, H. rubrisubalbicans* and *H.frisingense* cand. and for *Burkholderia brasilensis, Acetobacter diazotrophicus, Sinorhizobium meliloti* and for all *Azospirillum* species.

Up to now we used the new probes for the following applications: 1. Identification of nitrogen fixing isolates from different C4 fibre plant (*Miscanthus sinensis, M. sacchariflorus, Pennisetum purpureum* and *Spartina pectinata*), banana and tomato roots. 2. *In situ* monitoring of nitrogen fixing bacteria in inoculated sugar cane and *Miscanthus sinensis* plants under axenic and field conditions. 3. Observation of the early adhesion (1h to 8h after inoculation) of *Azospirillum brasilensie* and/or *A. lipoferum* to the root surface of axenic maize plants. 4. Assessment of the selective enrichment of diazotrophic bacteria from banana roots in different semisolid media by *in situ* hybridization.

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CHARACTERIZATION OF NEW HERBASPRILLUM LIKE ISOLATES FROM PENNISETUM PURPUREUM AND SUGAR CANE SAMPLES CAPTURED BY IMMUNOFISHING

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1. Introduction

It is well known that the biodiversity of microbes is largely underestimated. Therefore, efforts to identify not yet described new bacteria from different habitats are profitable. The conventional methods for the isolation of diazotrophic bacteria in plant and root samples have three distinct phases: (1) selective enrichment in semisolid N-free medium, (2) isolation of pure strains, and (3) confirmation of the diazotrophic nature by a variety of biochemical and molecular tests. In general, each of these phases takes more than 72 h to perform. Biological nitrogen fixation is one of the key processes in the global nitrogen cycle. Mainly in sustainable, agricultural used ecosystems its importance should be increased. To examine the diversity of *Herbaspirillum,* a nitrogen-fixing bacterium, in sugar cane and *Pennisetum purpureum,* we used immunofishing with specific polyclonal antisera to enrich *Herbaspirillum* from plant material. This technique is much faster than the conventional isolation process and independent from selective cultivation.

2. Material and Methods

The immunofishing approach was performed using protein A covered microtiterplates, coated with a mixture of a *Herbaspirillum seropedicae* and *Herbaspirillum rubrisubalbicans* specific antiserum to separate and concentrate *Herbaspirillum sp.* The enriched bacteria were detached from the microtiterplates using a glycin buffer with a pH of 3. This is followed by a standard isolation procedure. The enrichment factor was about 5000. The new isolates were characterized on the level of genotype (ERIC - PCR pattern) and phenotype (ELISA and periplasma-protein pattern) and compared to the known isolates by cluster analysis.

3. Results and Discussion

Using the immunofishing technique new *Herbaspirillum* isolates were obtained from sugar cane and *Pennisetum purpureum* samples. The intraspecies diversity of the isolates was very low. According to ERIC-PCR 3 different groups of isolates (2 groups from *Pennisetum,* 1 group from sugar cane) were obtained. Surprisingly none of the new isolates was identical with a known strain. On the basis of ERIC PCR and periplasma protein pattern, the isolates from sugar cane clearly fall into the *H. rubrisubalbicans* cluster, which confirms with earlier data using classic isolation techniques. The closest relative was strain *H. rubrisubalbicans* Ml, which was isolated from leaves of sugar cane in Mauritius. Using the same fingerprint techniques, the isolates from *Pennisetum purpureum* did not show high similarities neither to *H. seropedicae* nor to *H. rubrisubalbicans* strains. On the basis of 16S rDNA data all 3 groups of isolates show high homology to the *H. seropedicae-H. rubrisubalbicans* cluster.

PROMOTION OF THE GROWTH OF RICE BY INOCULATION OF NITROGEN-FIXING-ACTIVITY-ENHANCED BACTERIA TO THE RHIZOSPHERE

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Biological nitrogen fixation is important for sustainable agriculture by saving energy consumption. Rhizobia in the nodules of leguminous plants are estimated to fix approximately 80 million tons of atmospheric nitrogen per year on the earth, and even diazotrophs in the rhizosphere of non-leguminous plants, such as rice, fix about 10 million tons. The final purpose of our research is to improve the nitrogen fixing ability of bacteria in the rice rhizosphere, to save chemical fertilizer, and thus to save energy.

Several diazotrophs live in the rhizosphere of rice. Among them, we selected *Klebsialla oxytoca* and *Azospirillum lipoferum.* The former fixes nitrogen under anaerobic conditions and uses sugars as the main carbon source to make energy. Sugars are consumed by fermentation, producing organic acids. In contrast, the latter fixes nitrogen under microaerobic conditions, utilizing preferably organic acids as the carbon source. As the amount of carbon source excreted from rice root is limited, we suppose a combination of those two diazotrophs is important for improving nitrogen fixation in the rhizosphere.

By gene manipulation, we have developed *K. oxytoca* R16 and *A. lipoferum* TA1 which fix nitrogen even in the presence of high concentrations of ammonia (15 mM and 40 mM, respectively). In the *K. oxytoca* R16, *nifA* gene was modified to be transcribed constitutively and *nifL* gene was destroyed (Y.-M. Kim et al., 1989). *A. lipoferum* TA1 was made by transposition of a Tn5 derivative, in which the *nifA* gene of *K. oxytoca* is constitutively expressed by read-through from the kanamycin resistance gene. This strategy depends on our finding that the NifA of *A. lipoferum* activates the *nifH* promoter of *Azospirillum* under microaerobic conditions but not under aerobic conditions though the NifA of *K. oxytoca* activates it strongly both under aerobic and microaerobic conditions (T. Shigematsu et al., 1997).

We expect that these improved diazotrophs should be a good "biofertilizer" when applied to the rice rhizosphere growing on the paddy field fertilized with a low amount of nitrogen fertilizer. To prove their ability, seedlings of rice were planted on vermiculite (200 ml) with a rice culture medium (200 ml) containing various amounts of ammonium nitrate and cultivated for approximately one month in a phytotron under conditions of 14-hr photoperiod (28□C) and 10-hr dark period (22°C). The full concentration of ammonium nitrate was set to 1 mM, and the level was decreased to a half, a quarter, or one tenth of the full concentration. The lower amount of nitrogen element led to the less growth of rice. By combined inoculation of *K. oxytoca* R16 and *A. lipoferum* TA1 cells (both were approximately 0.1 g in wet weight), harvested from 10 ml of full growth culture, to the rhizosphere of the rice seedlings, their growth was promoted, and the heights of the plants cultivated with bacteria-inoculated media of one tenth or a quarter level of nitrogen element surpassed those of the rice cultivated with a quarter or a half level of nitrogen element without bacterial inoculation, respectively.

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MICROSCOPICAL STUDY OF THE SYMBIOSIS BETWEEN TAGASASTE AND *BRADYRHIZOBIUM* SP. (*CHAMAECYTISUS*): A NEW INFECTION BY CRACK PRODUCING INDETERMINATE NODULES.

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Tagasaste, *Chamaecytisus proliferus* (L.fil.) Link subsp *proliferus* var *palmensis,* is a legume endemic to the Canary Islands, that forms effective, indeterminate nodules in response to infection by strains of *Bradyrhizobium* sp. (*Chamaecytisus*) (León-Barrios et al 1991; Santamaría et al 1997). The aim of this work was to document the developmental morphology of the root infection process in root nodulation of tagasaste by two strains of *Bradyrhizobium* sp. (*Chamaecytisus*). Tagasaste formed indeterminate nodules, in which the first stages of the infection process appeared to include aborted infection of deformed root hairs.

Transmission electron microscopy confirmed the absence of infection threads in the nodule structure and showed that both strains of rhizobia invaded tagasaste nodules by the crack mode of entry followed by their dissemination through intercellular spaces and penetrated the plant cells directly through the structurally altered cell walls. A unique feature of crack entry in nodulation of tagasaste is that it is not restricted to lateral root axils, unlike normal infection in *Sesbania, Arachis, Stylosanthes* and *Neptunia* (Noyde et al 1994; Rana et al 1995; Chandler, 1978; Chandler et al 1982; Subba-Rao et al 1995). The early detection of nodule primordia in the inner cortex of the root suggests that the strategy used by these rhizobia to produce wounds to penetrate, is similar to the one used by bradyrhizobia to infect the non-legume *Parasponia.* In this case, the rhizobia produces cell proliferation of outer cortical tissue disrupting the peripheral cell layers allowing the bacteria to enter (Lancelle et al 1984). It seems that BTA-1 and BGA-1 can not penetrate the intact epidermis, but nevertheless make sufficient Nod factors, to stimulate the cortical root division. Presumably the emerging nodule splits the epidermis, creating the portal of entry through which the bacteria can access the nodule surface and enter via cracks. Although the tagasaste symbiosis involves crack mode of entry it forms indeterminate nodules with the typical cylindrical shape and gradient of cells at different stages of development. This is the first description of the development of an indeterminate nodule using this mode of infection.

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A SURVEY OF THE DIAZOTROPHIC POPULATION IN ASSOCIATION WITH MAIZE

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Previous work has shown that nitrogen-fixing bacteria in association with maize can reduce significant amounts of acetylene and/or provide growth benefits (Berge et al. 1990; Palus et al. 1996; Von Bulow, Dobereiner 1995), although the mechanisms responsible for these benefits are poorly understood. In this work, a survey of the endophytic population of diazotrophs was performed to identify patterns of diazotroph colonization of maize in response to various levels of nitrogen fertilization. Of the several nitrogen-fixing species that were cultured from plants receiving none to intermediate levels of N fertilizer, *Klebsiella pneumoniae* was most commonly isolated. The area of bacterial localization and dinitrogenase reductase (NifH) production within plant tissue was determined for two strains of *K. pneumoniae.* Bacteria were tagged with green fluorescent protein, inoculated onto several maize cultivars, and visualized using laser scanning confocal microscopy. *K. pneumoniae* inhabited intercortical layers of the stem and the region of maturation in the root. NifH production by bacteria growing within plant root tissue was evaluated using immuno-fluorescence staining. NifH production occurred by bacterial colonies residing on and within the root surface. Production of NifH was only evident when the plants were grown in a medium containing sucrose (0.1%). The expression of NifH by this species when associating with maize suggests that this may be a naturally occurring phenomenon if plant exudates or soil organic carbon meet the bacterial energy requirements for nitrogen fixation. These results, in addition to investigations on the activity of diazotrophs within maize tissue, should illuminate those interactions that can be further explored and manipulated to yield viable, productive associations of agronomic significance.

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SECTION IX

ACTINORHIZAL ASSOCIATIONS

FRANKIA AND ACTINORHIZAL PLANTS

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In actinorhizal symbiosis, actinomycetous soil bacteria of the genus *Frankia* induce the formation of nitrogen-fixing root nodules on dicotyledonous plants from eight different families (Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae and Rosaceae), usually woody shrubs. Phylogenetic evidence has shown that all plants able to form nitrogen-fixing root nodules, i.e. actinorhizal plants and legumes, map in the same clade and thus go back to a common ancestor (Soltis et al. 1995). More detailed studies by Swensen (1996) and Doyle (1997) have revealed that among the plants with this predisposition, both actinorhizal and rhizobial symbioses seem to have been established several times independently.

Frankia strain taxonomy has always been hard to access, particularly because the microsymbiont of several symbiotic interactions (Coriariaceae, Datiscaceae, Rhamnaceae and Rosaceae) has not been cultured yet (Lechevalier, Lechevalier 1990). Fortunately, the introduction of molecular ecology, using rDNA, *nif* or *glnII* gene sequences and PCR-based techniques for strain identification without culturing, has led to considerable progress in this area and allowed the determination of genomic groups including non-culturable strains (Normand et al. 1996; Ramirez-Saad et al. 1998; Cournoyer, Lavire 1999) and the analysis of genetic diversity of *Frankia* strains in natural habitats, including non-infective strains (Maunuksela et al. 1999; McEwan et al. 1999; Wolters et al. 1999). For instance, it has been shown that actinorhizal nodules can contain in their outer layers additional non-nitrogen fixing actinomycetes that cannot reinfect the host plant on their own (see e.g. Mirza et al. 1994; Ramirez-Saad et al. 1998). Furthermore, recently microsymbionts of the Rhamnaceae have been cultured and included in cross infection studies (Chaia 1998; Clawson et al. 1998; Caru, Cabello 1999; Ritchie, Myrold 1999). Recent progress on *Frankia* physiology includes the analysis of DNases and uptake hydrogenase produced by *Frankia* (Tavares, Sellstedt 1997), siderophore formation by *Frankia* strains (Arahou et al. 1998) and studies on antibiotic resistance (Dobritsa 1998; Caru, Cabello 1999).

Studies on the molecular biology of actinorhizal plants, mostly regarding the expression of nodule-specific and nodule-enhanced genes, have meanwhile been performed on *Alnus* (see e.g. Goetting-Minesky, Mullin 1994; Ribeiro et al. 1995; Pawlowski et al. 1997), *Casuarina* (Franche et al. 1998a), *Datisca* (Okubara et al. 1999) and *Elaeagnus* species (Kim 1998). To provide tools for gene expression studies in the course of nodule induction and development, transformation protocols have been developed for the actinorhizal trees *Casuarina glauca* and *Allocasuarina verticillata* (reviewed by Franche et al. 1998b). In preparation for plant transformation, attempts to set up regeneration systems have been made for *Datisca glomerata* and *Myrica gale* (Wang and Berry 1996; Tavares et al. 1998). Studies on transgenic plants containing nodulin promoter-GUS fusions enable the comparison of cell differentiation in actinorhizal nodules from different plant families as well as legume nodules. Using this approach, it has already been shown that the transcription factors activating the expression of (leg)hemoglobin are conserved between actinorhizal *Casuarina* and legumes (Jacobsen-Lyon et al. 1995; Franche et al. 1998a) and that infected and uninfected cells of *Casuarina* prenodules share the differentiation of their counterparts in nodules (Laplaze et al.

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1999a). Cytological analysis of actinorhizal plants has led to a better understanding of the function of flavan-containing cell layers in nodules (Laplaze et al. 1999b).

As in legumes, nodule induction by *Frankia* is subject to autoregulation by the plant host. Recently, detailed studies on autoregulation of actinorhizal nodule formation have been performed on *Alnus glutinosa* (Wall, Huss-Danell 1997), and furthermore, it has been found that phosphate nutrition, which has already been shown to affect actinorhizal nodule morphology (Burgess, Peterson 1987), has a significant influence on the autoregulation of nodulation in intracellularly (*Alnus)* as well as intercellularly *(Hippophae*) infected actinorhizal plants. The current progress in actinorhizal research leads to the hope that major breakthroughs, like the isolation of the *Frankia* Nod factor (Ceremonie et al. 1998) and the culturing of the microsymbionts of Rosaceae, Coriariaceae and Datiscaceae and their inclusion in cross-infection studies, may be achieved within the next years.

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SUGAR PARTITIONING IN NITROGEN-FIXING ROOT NODULES

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Nitrogen-fixing root nodules represent strong carbon sinks. In plants, carbon sources are transported in the form of sucrose from the source organs (mature leaves) via the phloem to the sink organs. Phloem unloading and post-phloem transport in the sink organs can proceed symplastically via plasmodesmata or apoplastically via sugar translocators (Fisher, Oparka 1996). During lateral root induction in Arabidopsis, phloem unloading occurs symplastically (Oparka et al. 1995) and symplastic continuity also exists between the phloem and *Agrobacterium tumefaciens*-induced tumors of *Ricinus* (Pradel et al. 1999). However, treatment of Arabidopsis with *Pseudomonas* elicitor or fungal pathogens leads to induction of sugar transporter expression, i.e. to apoplastic sugar transport (Truernit et al. 1996). The question arises how nodule bacteria -rhizobia or *Frankia* strains- induce phloem unloading while infecting their host plants, and how phloem unloading and carbon partitioning take place in mature, nitrogen-fixing nodules of both legumes and actinorhizal plants. How is sucrose unloaded from the phloem and transported to the infected cells, and how it is introduced into nodule metabolism? Are the partitioning mechanisms common to all root nodule symbiosis? To answer these questions, we focused on three different symbioses, one *Rhizobium*/legume symbiosis (*Medicago truncatula/Sinorhizobium meliloti*) and two actinorhizal symbiosis whose host plants belong to different phlogenetic groups (*Casuarina glauca* and *Datisca glomerata*; Swensen, 1996). *C. glauca* and *D. glomerata* differ in the organization of infected cells in the nodule cortex (Hafeez et al. 1984; Berg, McDowell 1987).

In order to analyse apoplastic sugar transport, cDNA fragments of hexose and sucrose uptake translocators were amplified by RT-PCR using degenerate primers derived from interspecific protein sequence comparisons. The expression of the corresponding genes in roots and nodules was compared using RNA gel blot hybridization, and expression patterns in nodules were analysed by in situ hybridization with antisense RNA. Furthermore, cells whose apoplast is blocked by secondary cell wall modifications, allowing only symplastic transport of metabolites, were identified cytologically. To examine how sucrose is introduced into nodule metabolism, the activity of sucrose-degrading enzymes - sucrose synthase and apoplastic, cytosolic and vacuolar invertases - was compared in roots and nodules. cDNA fragments of sucrose synthase and apoplastic invertase were amplified, and the expression patterns of the corresponding genes in nodules were analysed using in situ hybridization.

Our results showed high expression levels of hexose and sucrose translocators in nodules of *M. truncatula,* as well as high apoplastic invertase activities. These data indicate that apoplastic transport plays an important role in the vascular system as well as the inner tissue of mature legume nodules. In actinorhizal nodules, however, post-phloem transport seemed to occur mostly symplastically (with the exception of transport across the vascular endodermis in C. *glauca* nodules). These results are convincing in so far that in order to control the symbiotic bacteria, the plant has to be able to limit their access to nutrients. In actinorhizal nodules, *Frankia* is not taken up into the host cells by a complete endocytotic process, but stays in branching infection thread-like

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structures. Thus, the perisymbiont space is continuous with the apoplast, and accordingly, apoplastic sugar transport in the nodule cortex might allow the microsymbiont free access to photosynthates. In contrast, in most legume nodules analysed thus far, including those of *M. truncatula,* rhizobia are internalized in infected cells via a complete endocytotic process (reviewed by Mylona et al. 1995). Hence, any nutrients have to be actively transported over the peribacteroid membrane to reach the intracellular bacteroids. This allows the plant full control of the microsymbiont's access to carbon sources. In this context, the question arises whether apoplastic sugar transport in nodules is a feature common to all legume symbiosis, or whether in legume nodules with persistent infection threads (reviewed by Sprent 1989), only symplastic sugar transport takes place in the inner tissue. Since it is generally assumed that rhizobial bacteroids are supplied by the host plant with dicarboxylates, not sugars (Streeter 1995), it is possible that bacteroids do not express sugar uptake systems and thus cannot use the plant's apoplastic sugars.

The carbon source delivered by the host plant to nitrogen-fixing *Frankia* has yet to be determined. Preliminary evidence suggests that like in legume symbioses, dicarboxylates are fed to the bacteria (Akkermans et al. 1983). However, our studies on hexose translocator expression in *Datisca* nodules imply that at least in this system, the bacteria may be supplied with hexoses. Further studies are needed to clarify this point.

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FLAVAN-CONTAINING CELLS DELIMIT *Frankia* INFECTED COMPARTMENTS IN *Casuarina glauca* NODULES

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1. Introduction

Flavonoids are secondary metabolites derived from the phenylpropanoid pathway. They are implicated in a wide range of biological phenomena such as, establishment of pathogenic and symbiotic plant-microbe interactions, modulator of polar auxin transport, pigmentation of flowers, and biotic/abiotic stress resistances (Shirley, 1996). Chalcone synthase (CHS) is the first enzyme of flavonoid biosynthesis catalysing the condensation of one molecule of malonyl CoA with 4-coumaroyl CoA to produce chalcones. Several authors have suggested that CHS is a key regulatory enzyme of the flavonoid pathway and that CHS gene is a good molecular marker of flavonoid production (Hahlbrock, Scheel, 1989).

There are numerous studies showing flavonoids to be of major importance in symbiotic interactions between legume plants and rhizobia (Schultze et al. 1994). However, there are only few reports of the possible role of flavonoids in actinorhizal plants-*Frankia* interactions. We are working on *Frankia-Casuarinaceae* symbiosis as a model family to study actinorhizal symbioses. The aim of this work was to investigate the involvement of polyphenols in the *Casuarina glauca-Frankia* symbiosis. We characterized histochemically and by HPLC the major polyphenol compounds and monitored CHS gene expression in *C. glauca* nodules. We report that in this symbiosis *C. glauca* nodules polyphenol compounds belong to the flavan group of flavonoids and we proposed possible functions of flavans in the *C. glauca-Frankia* interactions.

2. Results and Discussion

2.1. Phenolic containing cells create a compartmentation of *C. glauca* nodule lobes

The root nodules of *C. glauca* are composed of closely packed nodule lobes with uninfected roots emerging from the tips of each lobe. Each nodule lobe is a modified lateral root without root cap, including central vascular tissue, cortical parenchyma infected with *Frankia* and a superficial periderm (Franche et al, 1998). Longitudinal sections of mature nodule lobes stained with toluidine blue showed an accumulation of phenolic compounds in few cell layers below the periderm, in the endoderm and at the boundary between the apex of the nodule lobe and the nodule root. In the cortical tissue, cells containing phenolics formed continuous files from the apex to the base of the nodule lobe. Thus, cells containg phenolics limit different cortical areas of infected and uninfected cells.

2.2. Phenolics compounds belong to the flavan class of flavonoids

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Several tests were used for the histochemical analyses of phenolic compounds on sections of nodule lobes. For example, positive reactions were found for both vanillin-HCl (Sarkar, Howarth 1976) and DMCA (Feucht, Treutter 1990) reagents in all cells containing polyphenols, suggesting that flavans are present in these cells. Soluble phenolics were further analysed using reversed-phase chromatography in a HPLC system. The elution profiles of the extracts prepared from mature nodules showed ten major peaks. Spectra data indicated that two peaks were not phenolic compounds and that all other peaks corresponded to compounds belonging to the flavan class of flavonoids. Based on their retention time, co-injection experiments and absorption spectra two peaks were identified as (+)-catechin and (-)-epicatechin. To compare flavonoid metabolism in nodule and uninfected root the same HPLC analysis was conducted with root extracts. This analysis indicates that the same eight major flavan compounds were present in roots as in nodules but in lower amount. Moreover, there were changes in the relative proportion of products indicating a reorientation of the metabolic fluxes in the different biosynthetic pathways

2.3. CHS gene is expressed in the flavan-containing cells of the apex of the nodule lobe

We used a *C. glauca* CHS cDNA as a probe to localize CHS transcripts in *C. glauca* nodule lobe by *in situ* hybridization. CHS transcripts were detected in flavan-containing cell layers between the nodule lobe and the nodule root, below the periderm, in the endoderm, and in the apical part of the cortical cell files. Thus, the expression of CHS was correlated with the detection of flavans in the same cell type. However, CHS expression was rectricted to the apical part of the cortical flavonoid-containing cell layers, suggesting that CHS mRNA appeared transiently at the apex and that enzymes of the flavonoid pathway are still active in cell derivative within the cortex to produce the tannin-containing cell layers. This hypothesis can be tested using antibodies directed against CHS.

3. Conclusion

We have found that *Frankia* infection leads to a dramatic increase of flavans biosynthesis in nodules compared to uninfected roots. We have shown the existence of regular pattern of cell layers containing flavans. Besides the flavan accumulation below the peridem, in the endoderm and between the nodule root and the apex, we have shown accumulation in cell layers of the cortex limiting compartment of infected and uninfected cells. The biological significance of this compartmentation might be to prevent the spread of the endophyte in the vascular bundle and in the nodule root. Alternatively, the flavan layers might contribute to the protection of secondary infection and/or the limitation of oxygen penetration in the nodule.

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CASUARINA-FRANKIA SYMBIOSES – MOLECULAR STUDIES OF *FRANKIA*

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1. Introduction

Frankia commonly have uptake hydrogenase (hup), an enzyme oxidizing hydrogen evolved from nitrogenase. Only one *Frankia* up to date has been recorded to be phenotypically hup-, i.e. lacking hup (Sellstedt et al. 1986). Hydrogenases are enzymes occurring mainly in procaryotic organisms, but also in a few eukaryotic organisms. Five different hydrogenases have been found (Wu, Mandrand, 1993). Regulation of *hup* by hydrogen has been reported in B*radyrhizobium* j*aponicum* (Maier et al., 1979, Kim et al., 1993) but is not known in detail in *Frankia.* Regulation of hup is important as well as localization, and earlier studies of the localization have revealed presence of the enzyme in vesicles as well as in hyphae (Sellstedt, Lindblad, 1990).

Transformation of *Frankia* would be useful e.g to create *hup* mutants, but transformation has so far not been reported. Interestingly, a large release of DNases (Tavares, Sellstedt, 1997) as well as DNA (Tavares, Sellstedt, 1999a) have been reported from *Frankia* strains R43 and HFPCcI3. The DNases released from *Frankia* were actively degrading all DNA except *Frankia* DNA released to the medium and that was adsorbed to the cell wall. Studies of the release of DNA and DNases may increase the probability of developing techniques to promote gene transfer. Release of DNA into the environment has been reported before (Lorenz, Wackernagel, 1992) and is considered as a source of nitrogen, carbon and phosphorous.

2. Material and Methods

Frankia strains HFPCcI3 and R43 isolated from *C cunninghamiana* were grown in BAP (Fontaine et al. 1986), while KB5 isolated from *C. equisetifolia* was grown in PUM (i.e P-medium (Burggraaf, Shipton, 1982) with 1/10 ionic strength). Hup was measured by use of a hydrogen electrode (Sellstedt, Winship, 1987). Immuno-gold labeling was performed as described in Sellstedt, Lindblad (1990) as compared to Wheeler et al. (1997). Preparation of cell wall (CW) and extra-cellular (EC) fractions were performed according to Tavares, Sellstedt (1997). DNase activities of these different fractions were checked in agarose gels and by renaturing SDS-PAGE (Tavares, Sellstedt, 1997). The mobility gel band shift assay was performed by use of a non-denaturing 4% polyacrylamide gels. Cell lysis was checked by ICDH-assays (Tavares, Sellstedt, 1999b). Origin of the DNA was tested by Southern blot hydridization.

3. Results and Discussion

Uptake hydrogenase activity followed the same pattern as nitrogenase activity but reached maximum value one day later. Correlation between nitrogenase activity and hydrogen uptake has been shown in several nitrogen-fixing organisms before (e.g. Graham et al 1984). Nitrogen fixation is an energy demanding process, but no effect on nitrogenase activity could be seen in *Frankia* KB5 after addition of 10% dihydrogen for four hours in cells grown in either carbon or carbon limiting condition. However, after 24 hours of carbon limitation the effect on nitrogenase activity was intriguing with no activity recorded. Interestingly, some activity could be restored by addition of dihydrogen. In western blot analysis when using antibodies produced against the Fe-protein of nitrogenase we could see that the nitrogenase was present after 24 hours in carbon-depleted media irrespective of dihydrogen was added or not. This result implies that the Fe-

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protein subunit of nitrogenase at this stage was not broken down due to higher internal oxygen concentration caused by lower respiration rate as discussed by Murry and Lopez (1989). The loss of nitrogenase activity was rather due to lack of an energy source at this stage. Earlier results have shown a distribution of hydrogenase in vesicles and hyphae (Sellstedt, Lindblad, 1990). By use of freeze-fractionation we improved the immunogold localization technique (Wheeler et al. 1997). Hereby we were able to show that *hup* is mainly localized in the vesicles and to a lesser extent in the hyphae.

DNases were present in both *Frankia* CW and EC fractions, as shown before (Tavares, Sellstedt, 1997). In spite of this, we also show here that DNA was present in both fractions, and surprisingly, this DNA was resistant to the DNases released from the strains. The DNA found was secreted during the exponential growth phase, however, a small degree of cell lysis was occurring. Using the CW-DNA as a probe under high stringency conditions it was shown that the DNA was of a chromosomal origin. Moreover, in order to explain the intriguing co-existence of DNA and DNases we performed mobility gel band shift assays, which showed that the DNA was engaged in a complex with other molecules most probably proteins, contributing to the resistance of the DNA to DNases.

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DISTRIBUTION AND PHYLOGENY OF *Gymnostoma* SPP. INFECTIVE *Frankia* STRAINS IN NEW CALEDONIA

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Plants in symbiosis with *Frankia* belong to eight families of dicot plants, one of the most abundant being the Casuarinaceae. This tropical taxon comprises 4 genera: *Casuarina, Allocasuarina, Ceuthostoma* and *Gymnostoma.* The most represented is *Casuarina,* the origin of which is Australia but *C. equisetifolia* now has a world-wide distribution thanks to **17-18th** century English sailors who transplanted it because it grew well on beaches and could provide boat construction wood. The first *Frankia* strains isolated from *Casuarina* were of two types: one "parasitic" unable to fulfill Koch's postulates but able to infect another host plant, namely the Elaeagnaceae *Hippophae,* another made up of strains infective on *C. equisetifolia* (Diem et al., 1983). The second group of strains was well characterized in 1983 by Diem et al. and found to belong to a cross-inoculation group all of its own. The phylogenetic position of these closely related isolated strains from all over the world was determined by analysis of the *rrs* genes in 1991 by Nazaret et al. to be very close to that of *Frankia alni,* one of the genomic species comprising strains infective on *Alnus.* Later on, Rouvier et al. (1996) analyzing the unisolated symbionts of a number of *Casuarina* and *Allocasuarina* species growing in their natural place of origin, Australia found that each species had one or exceptionally two specific micro-symbionts, none of which could be cultured. This brought the idea of a co-speciation of plants and symbionts.

Gymnostoma is a plant genus that appears to have evolved in the eastern part of Gondwana, away from the drier Australia where *Casuarina* and *Allocasuarina* evolved. The genus comprises 16 species, 8 of which are endemic to New Caledonia. This island situated 1000 km North-East of Australia is a hot spot of diversity. It is made up of a ultramafic substratum rich in nickel and poor in magnesium and other nutrients. The aims of our work were 1) to determine if preliminary reports made on two strains isolated from *Gymnostoma* and described as close to Elaeagnaceae-infective strains were representative of field collected nodules or represented "parasitic" non-symbiotic strains and 2) to determine if the *Frankia* strains diversity was related to host plant and to soil type. Primers targeting a *Frankia*-specific region at coordinates 980 of the 16S gene were used to amplify the whole of the IGS and 500 nt of the 5' part of the 16S gene. With *Casuarina* and *Allocasuarina* nodules, this yielded a sequence identical or very close to that of the *Frankia* strain infective on *Casuarina.* When done on *Gymnostoma,* amplicons were obtained that were identical to that of D11, an Elaeagnaceae-infective strain. This result was confirmed when the *nifDK* region was amplified and sequenced. This region is highly variable and we have built a significant sequence data base. The amplicons obtained from a number of *Gymnostoma* nodules were in the group of Elaeagnaceae-infective *Frankia* and away from other genomic groups. Another confirmation was that isolated strains fulfilled Koch's postulates and could not induce nodules on *Casuarina.*

Morphological observations of isolated strains and cross-inoculation trials showed that *Gymnostoma*-infective strains are indistinguishable from *Elaeagnus*-infective strains, in particular on the basis of pigments production, size of sporangia (small) and shape of hyphae (contorted).

Thus, in spite of the fact that the family Casuarinaceae is coherent morphologically and phyletically as determined by Maggia and Bousquet (1994) by sequencing the nuclear ribosomal spacer, the

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symbiosis appears to have evolved allopatrically in two divergent directions, on the one hand *Casuarina* and *Allocasuarina* with more and more specific symbionts, on the other hand *Gymnostoma* with Elaeagnus-infective symbionts.

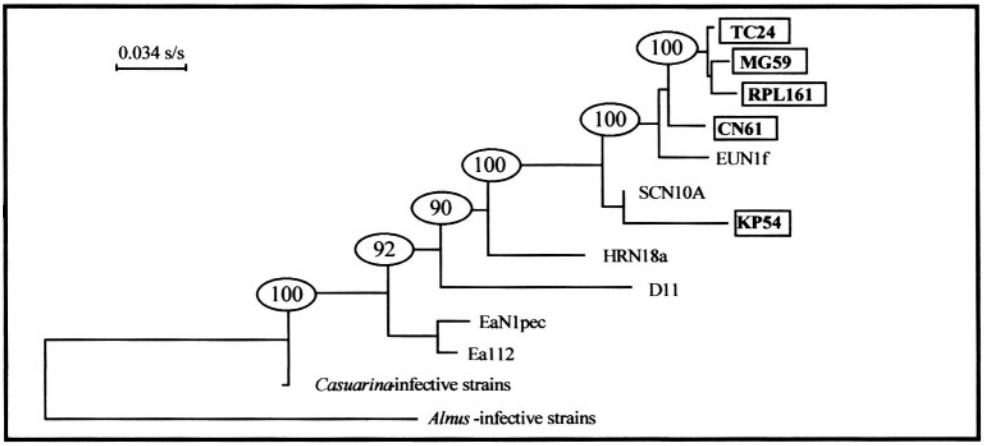

Fig. 1. Phylogenetic Neighbor-Joining tree based on *nifD-K* intergenic spacer sequences of *Gymnostoma* symbionts (boxed). The bar represents 0.034 substitution per site. The numbers in ovals are percentages of bootstrap replicates in which the cluster was found (Navarro et al., 1997).

To determine what structured the diversity of *Frankia* strains in New Caledonia, a study of 358 field-collected nodules from the 8 endemic *Gymnostoma* species growing on the 4 main soil types present in New Caledonia was made. It showed that contrary to the situation in the *Casuarina* and *Allocasuarina* genera where a strict host plant specificity had been found, (one plant species, one *Frankia* type), no specificity could be found. A multiple correspondence analysis nevertheless showed that the distribution of these patterns was related to soil type and to host-plant species (Navarro et al., in press). Furthermore, several *Frankia* strains were exclusively associated with the ultramafic soils. For instance, *G. webbianum* is the only species found in both the acidic alluvium and in the ultramafic soil. However, the patterns associated with that host plant are first and foremost associated with the soil. In other words, it appears that the soil type influences on the one hand the host plant because different *Gymnostoma* species have different soil preferences. On the other hand, *Frankia* strains also have soil preferences and this appears to be the dominant factor. A physiological study of these strains has shown some of them to be able to grow in the presence of important amounts of nickel. As the amount of nickel increases, the cells divert more of their metabolites to the synthesis of an extracellular matrix and less and less to cell division. The study of the mechanisms involved is under way.

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SPECIFICITY IN *DISCARIA* - *FRANKIA* SYMBIOSES

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For some years we have been studying symbioses in native actinorhizal plants belonging to the Family Rhamnaceae, namely *Discaria trinervis, Discaria chacaye* and *Discaria articulata.* All these species are distributed in the south west of South America, at the Patagonia. We are interested in the mechanisms of regulation of the nodulation and the meaning of specificity and recognition in these symbioses, in order to look for the unknown symbiotic signals involved in the *Frankia* actinorhizal association.

We have succeeded to isolate *Frankia* from field nodules and to cultivate them *in vitro* from field nodules of the three plant species. *Frankia* BCU110501 was the first infective and effective isolate from *Discaria trinervis* (Rhamnaceae) that was published (Chaia, 1998). Recently, *Frankia* BCU110345 has been isolated from field nodules *of Discaria chacaye,* and *Frankia* BCU110601 has been isolated from field nodules of *Discaria articulata.* The three isolates were infective and effective, producing nitrogen fixing nodules *in vitro* after inoculation on its original host plants.

The infection and nodule development in *Discaria trinervis - Frankia* BCU110501 was studied as a model system using the pouches growth method. Root infection occured via intercellular penetration. Looking for the time course of nodule development we found that nodule primordia developing from perycicle could not be seen at day 3 after inoculation (a.i.) but were present at day 6 a.i. ; hyperplasic nodule cell infection by *Frankia* was clear at day 9 a.i. and whole *Frankia* differentiation was hystologically complete at day 16 a.i. (Valverde & Wall, 1999a). Nitrogen fixation, measured as ARA, could be detected after day 21 a.i. (Valverde *et al* 1999). By experiements with delayed re-inoculation of the tap root and split root systems, we could demonstrate that infection and nodule development in *Discaria trinervis* was quickly feed-back inhibited after intercellular root penetration by *Frankia,* but before plant cell infection by bacteria filaments; nodule development was arrested at nodule primordia stage before nodule cells were infected by *Frankia.* Afterwards, further infection and nodulation was still inhibited by mature fixing nodules and regulation of symbiotic activity appeared to be controlling the efective nodule biomass development (Valverde and Wall, 1999b).

To characterize the host range of specificity of *Frankia* isolates from Rhamnaceae we tested isolate BCU110501 on different actinorhizal plants. We inoculated seedlings belonging to three of the four clades described in the filogenetic tree proposed by Swensen & Mullin (1997), based on analysis of chloroplast gene rbcL and different symbiotic features. Appart from effectively nodulating its original host, this isolate from *Discaria trinervis* was infective and effective on *D. chacaye*, *D. articulata, D. americana, Trevoa patagonica, Colletia hystrix, Hippophaë rhamnoides* and *Elaeagnus angustifolia,* but was not able to induce nodules in *Ceanothus thrysiflorus x leucodermis* although this one beeing a Rhamnaceae plant. Nevertheless, the result is in agreement with 16S rDNA sequences analyses of different Frankia strains extracted from nodules of plants belongin to the Families Rhamnaceae and Elaeagnaceae (Clawson et al 1998). These authors found that all *Frankia* tested from Rhamnaceae and Elaeagnaceae plants shared a common symbiotic origin except those

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from *Ceanothus americanus* nodules which appeared to be more related to Rosaceae *Frankia* strains.

Frankia BCU110501, isolated from *D. trinervis,* was not able to infect and nodulate *Datisca glomerata, Alnus incana, Alnus acuminata* and *Casuarina cunninghamiana,* which are species belonging to two other plant clades of the filogenetic tree proposed by Swensen and Mullin (1997). After these results we can propose that, from the plant side, it seems that there is a signal compatibility for symbiotic recognition within actinorhizal plants evolved within the same proposed clade. When we looked at recognition from the bacterial side, we tested different *Frankia* isolates from different actinorhizal plants inoculated on *Discaria trinervis* roots. Only *Frankia* isolates from *Discaria spp* or *Eleagnus angustifolia* - BCU110501, BCU110345, BCU110601, EaI5b - were able to induce nodules on *Discaria trinervis* roots while isolates from plants belonging to another clade - CpI1 isolated from *Comptonia peregrina,* ArI3 isolated from *Alnus rubra,* CjI82 isolated from *Casuarina japonicum,* Allo2 isolated from *Allocasuarina* - were not able to induce nodules on *Discaria trinervis* roots.

We asked the following question about cross inoculation groups, specificity, and recognition: Is there any degree of plant species-specificity between *Frankia* and roots within the same plant Family and same genus? To answer this question we inoculated the following *Frankia* isolates from *Discaria* species: BCU110501 (from *D. trinervis*), BCU110345 (from *D. chacaye*) and BCU110601 (from *D. articulata*) on each of the three plant species. We used the same amount of bacterial inoculum from the same batch. We inoculated all the plants simultaneously in order to compare nodulation kinetics. The rate of nodulation was followed using the pouches growth technique. Homologous symbiotic pairs show the best recognition indicated by faster infection and nodulation rates.

All toghether, these results suggest that similar but not identical molecular mechanism of recognition should exist within the plant clade of Rhamnaceae and Eleaegnaceae, probably showing minor molecular differences. Moreover, because of the very early feed-back inhibition mechanism of infection and nodulation that we have described for *Discaria trinervis* (Valverde and Wall 1999b), this degree of symbiotic specificity could be selected by the plant in a competitive situation within a diverse *Frankia* population.

This model of symbiotic interaction suggests that early interaction and recognition events between plant root and free living *Frankia* would select the best fitted symbiotic pair, in other words, the more specific interaction.

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NOVEL NODULE-SPECIFIC GLYCINE- AND HISTIDINE-RICH PROTEINS EXPRESSED IN THE ZONE OF INFECTION OF ACTINORHIZAL NODULES MAY BE MULTIMERIC METAL-BINDING PROTEINS.

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In the nitrogen-fixing actinorhizal symbiotic system the presence of a compatible *Frankia* strain alters the developmental pathway of host plant lateral roots in such a way that they become nitrogen-fixing root nodules capable of supplying the plant with reduced nitrogen. It has been shown that nodule development in actinorhizal *Alnus* is accompanied by the expression of genes not expressed in the roots, leaves or other parts of host plants. Two nodule-specific cDNAs have been isolated that have the capacity to code for the small putative metal-binding proteins AgNt84 and Ag164 (Pawlowski *et al.* 1997). These proteins each have a putative hydrophobic signal peptide of 26 amino acid residues and a glycine/histidine-rich domain that may be involved in metal binding. mRNA coding for these proteins has been detected only in cells that are in the process of being infected by *Frankia* and has not been detected in cells with active nitrogen fixation or in cells that have not come into direct contact with *Frankia.* In this paper strategies for protein expression and a preliminary characterization of expressed proteins are presented.

As earlier reported (Dobritsa, Mullin, 1996), attempts to express full-length AgNt84 protein fused with maltose-binding protein (MBP) in *E. coli* resulted in poor growth of transformed cells under conditions of induction. Only a truncated MBP-AgNt84 protein with the first 16 amino acids deleted could be reliably expressed, and this protein was not efficiently cleaved with Factor Xa. In a further attempt to express full-length AgNt84, an *in vitro* expression system developed specifically for the expression of toxic proteins was used. DNA containing the entire coding region of *agNt84* cDNA was cloned into plasmid pET9-a and was used to transform *E. coli* strain BL21(DE3)pLysS. This system is designed for tighter regulation of transcription from the T7 promoter in pET vectors by reducing or eliminating the expression of T7 polymerase under non-inducing conditions. Only a mixture of truncated AgNt84 proteins, cleaved at the following positions, Ala-26, Ala-30, or Ala-32 was expressed using this system and only with late and short periods of induction. Induction resulted in rapid and complete cell lysis. The putative signal peptide of AgNt84 is apparently extremely toxic to *E. coli* such that even expression systems designed specifically for the expression of toxic proteins can not accommodate this peptide fragment either located internally in a protein such as is the case with the MBP-fusion protein, or at the N-terminus.

In order to express AgNt84 and its homolog Ag164 in a stable and reliable manner, new constructs were made by engineering *Nde*I sites into the respective cDNA clones such that cleavage with *Nde*I resulted in a restriction fragment that did not contain any nucleotides coding for the putative signal sequence. These restriction fragments were cloned into pET9-a and the resulting recombinant plasmids were used to transform *E. coli* BL21(DE3)pLysS. Many

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transformants expressing truncated AgNt84 and Ag164 were obtained from these transformations, two of which, 84-6 and 164-20, were chosen to express proteins for purification. Purification of the proteins was accomplished by metal affinity chromatography using Probond Ni resin (Invitrogen).

Purified 84-6 and 164-20 proteins, when electrophoresed on 12% SDS–PAGE gels using standard Laemmli conditions, consistently migrated more slowly in gels than would be predicted by their calculated molecular weights. DNA sequence analysis of the recombinant plasmids giving rise to these proteins confirmed the correct initiation and termination sites in the clones, N-terminal amino acid analysis confirmed the correct N-terminal sequence, and mass spectral analysis confirmed the correct polypeptide composition, indicating that the correct polypeptides were being expressed. 84-6 migrated in 12% SDS–PAGE gels as two bands of approximately 22.5 kDa (the predominant band) and 17.7 kDa. Both bands have the correct amino acid N-terminal sequence indicating that one is not a contaminant. Given that the MW of the 84-6 polypeptide is 7.8769 kDa, the 22.5 kDa band is likely to represent a trimer and the 17.7 kDa band a dimer. When incubated overnight in 8M urea and electrophoresed on SDS–Urea gels, the migration of the bands is most consistent with a shift to dimer and monomer. 164-20, the smaller of the two polypeptides (6.415 kDa), migrates in 12% SDS–PAGE gels as two bands of approximately 26.3 kDa (the predominant band) and 18 kDa protein with the predominant band shifting to 18 kDa in the presence of 8M urea. This is consistent with the existence of tetrameric and trimeric forms of 164-20. Electrophoretic patterns obtained on non-denaturing gels are similar to those on SDS gels. These results, in combination with the inability of either 84-6 or 164-20 protein to pass through Centricon 10 membranes, are consistent with the occurrence of multimeric proteins.

Although other proteins have been found to contain glycine- and histidine-rich domains, and other proteins with similar signal peptides are known to exist, we are aware of no proteins that are homologous to AgNt84 and Ag164. Both 84-6 and 164-20 bind tightly to Probond Ni affinity resin and we predict that each polypeptide may have the capacity to bind 7 and 5 divalent metal ions, respectively. As trimeric or tetrameric proteins, this would increase the capacity of each protein to 21 and 20 metal ions, respectively. Current studies are focusing on determination of the subcellular location of these proteins and on their metal-binding affinities and specificities.

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ROOT NODULE INITIATION, GROWTH AND FUNCTION IN *Discaria trinervis* (RHAMNACEAE) AS AFFECTED BY EXTERNAL N AND P CONCENTRATIONS

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Nitrogen and phosphate may influence N_2 fixation in legumes and actinorhizal plants at various levels of the symbiotic interaction, i.e. infection and nodule development, nodule function and host plant growth (Marschner, 1995). We studied the effects of altering P supply, with or without N, on nodulation, nodule activity and growth of *Discaria trinervis* seedlings. Seedlings were grown in pouches. In one experiment, they were supplied with different phosphate and N (as NH_4NO_3) concentrations. In a second, only P concentration was varied. All seedlings were inoculated with *Frankia* BCU110501 cells at 33 days after germination (Valverde, Wall, 1999a; 1999b). At harvest time (10 weeks after inoculation), nodulation, nodule distribution, nitrogenase activity (as ARA) and growth (dry matter) were determined. In dried leaves, N content was measured by Kjeldahl-Nessler colorimetry and total P content by the molybdate-blue method after dry digestion.

When varying N and P levels, nodulation was inhibited at high N supply (100 ppm) and low or normal P supply (0.001 or 0.1 mM). High P (10 mM) reversed N inhibition of nodulation. Distribution of nodules in tap roots was not affected by any P level at nodulation-permissive N levels. The final number of nodules was independent of N and P supply for low or moderate N levels (1 or 10 ppm). Plant dry matter production, shoot dry matter allocation, and shoot height were stimulated by P supply at low or moderate N supply. Differences in plant growth were noted from 6 weeks after inoculation. Individual nodule size and leaf area showed a similar positive response to higher P supply. Allocation of dry matter to nodules was stimulated by P, at either low or moderate N supply. Thus, nodule growth was positively affected by P. As expected, both plant nitrogenase activity and foliar area were higher for higher P supply. Specific nitrogenase activity showed less variation with P supply, indicating that nodule N metabolism was less affected by P and N levels used. Leaf N content was the same for all P and N levels, but a higher P supply induced higher P concentrations in leaves. When varying P level only, again nodule initiation (timing of nodule formation), autoregulation (nodule distribution) and final nodule number were not affected by P supply (from 1 μM to 1 mM). Plant growth was impaired at low P supply (1 to 10 μM), stimulated for P in the range between 10-100 μM, and not affected by 100 to 1000 μM P. While plant dry matter increased by a factor of *ca.* 2.2 when P varied between 1 μM and 500 μM, nodule size increased by a factor of *ca.* 4. Dry matter allocation to nodules (rather than roots) was stimulated by P. Shoot height was augmented by P supply at all P levels after 5 weeks of inoculation. In leaves, N content was slightly increased with P supply, from 2.7 to 4% of dry matter. Our results indicate a positive effect of P on the symbiosis mediated by control of nodule growth, rather than initiation and functioning as was the case for *Alnus incana* (Wall *et al.*, 1998). Interaction of N and P on nodulation is likely to occur, since P was able to reverse N inhibitory effects on nodulation, though at non-physiological P levels.

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TRANSGENIC *CASUARINACEAE* AS A TOOL FOR THE STUDY OF ACTINORHIZAL SYMBIOSIS

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Considering the numerous physiological, biochemical and morphological studies that have been carried out on the genus *Casuarina,* and the numerous uses in tropical and subtropical agroforestry, we have chosen the *Casuarinaceae* family as a model to study actinorhizal symbioses.

Using the disarmed strain of *Agrobacterium tumefaciens* C58C1(pGV2260), transgenic plants of *Casuarina glauca* and *Allocasuarina verticillata* were obtained in our laboratory. Genetically transformed *Casuarinaceae* appear as a major tool to identify the regulatory mechanisms that control the expression of actinorhizal symbiotic genes, for dissecting the role of specific proteins in nodule developmental process using sense and antisense strategies, and for comparing regulatory mechanisms that control the expression of legume and actinorhizal symbiotic genes. Our progress in the analysis of a metallothionein gene expressed in *C. glauca* nodules will be presented.

EFFECTS OF BORON DEFICIENCY ON GROWTH, STRUCTURE AND FUNCTIONALITY OF A *FRANKIA* STRAIN

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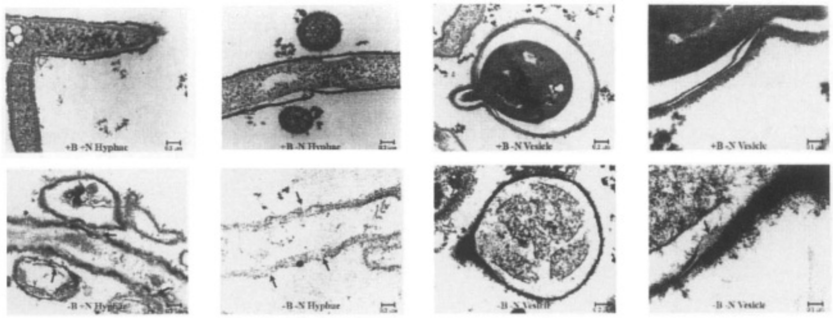
Frankia BCU1105 N_2-fixing, functionally analogous to the heterocysts of cyanobacteria (Zehr et al 1998). Because Boron (B) is essential for the stability of the heterocyst envelop and hence for the aerobic nitrogen fixation in cyanobacteria (Bonilla et al 1990), and some components of *Frankia* wall and vesicle envelops have the ability of cross-link with B, it is possible to theorize in a necessity of Boron for the maintenance of *Frankia* structures. *Frankia* BCU1105 was cultivated in BAP glucose medium with (+N) or without (-N) ammonium, to develop N_2-fixing vesicles in the latter, and in the presence (+B) or in the absence (-B) of B. Growth, tested as protein content, and the ultrastructure of hyphae and vesicles was studied. Cultures of *Frankia* BCU1105 did not grow in media without combined nitrogen (-N) and in the absence of B (-B), suggesting a requirement of the micronutrient for growth under N2-fixing conditions. The transmission electron microscopy study of cultures (figure) shows that in –B treatments the cytoplasm of hyphae was almost empty compared with controls (+B). The inner of B-deficient vesicles appeared fragmented. Also, the wall of B-deprived cells seems to be weakly packed, with a structure highly disorganized and disrupted at several points (arrows). Wall glycolipids (Tunlid et al 1989) and vesicle envelope hopanoids (Kleeman et al 1994) could make bridges with B during material deposition for growth and for vesicles differentiation, making B essential for *Frankia* structure and development. Besides the lack of B, damaged vesicles might not function, leading to a nitrogen starvation which could explain that *Frankia* do not grow in –B-N conditions.



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CELL WALL-ASSOCIATED PROTEINS OF *FRANKIA*

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The functional importance of the gram-positive cell wall as a distinct cellular compartment has been emphasized over the last years for several bacteria (Navarre, Schneewind, 1999). However, these studies have been hindered due to the particularly difficult preparation of cell wall extracts. Despite two methods of bacterial cell fractionation are well described in the literature (Fig. 1), isolation of the cell wall fraction is still problematic for many bacteria. Recently we optimized a procedure to extract cell wall-associated proteins from the symbiotic nitrogen-fixing actinomycete *Frankia* (Tavares, Sellstedt, 1999). The method was based on washing *Frankia* mycelia by repeated passage in-and-out of a micropipette tip for 3 min in 1 ml of 62.5 mM Tris-HCl (pH 6.8) buffer supplemented with 0.1% Triton X-100 as solubilizing agent. By this procedure peptidoglycan hydrolases were specifically detected in the cell wall protein fraction but not in the cytoplasmic protein extract, indicating a specific extraction of cell wall-associated proteins. These results support previously reported data showing a specific pattern of DNase activities for the cell wall extracts using the same strategy (Tavares, Sellstedt, 1997). The cell wall-associated protein fraction was efficiently extracted in less than 10 min, recovering approximately 94.5 ± 7.44 μg protein per extraction procedure, when high cell density cultures were used. Furthermore SDS-PAGE analyses of the cell wall extracts revealed an intriguing diversity of cell wall-associated polypeptides. The amount of cell lysis occuring during the cell wall extraction and assayed by isocitrate dehydrogenase activity (ICDH, EC 1.1.1.42), was estimated to be 1.50 ± 0.51%. Moreover, growth of the treated *Frankia* cells (i.e. cells from which the cell wall-associated proteins were removed) in semi-solid media, showed that the treated cells were still viable, which is important for studies of the functionality of cell wall-associated proteins. Stirring culture conditions seemed to be a necessary prerequisite to successfully extract cell wall-associated proteins by this procedure. This is likely to be due to the formation of microcolonies with an "open mesh" structure and also to a balanced hyphal growth as previously described (Schwencke, 1991).

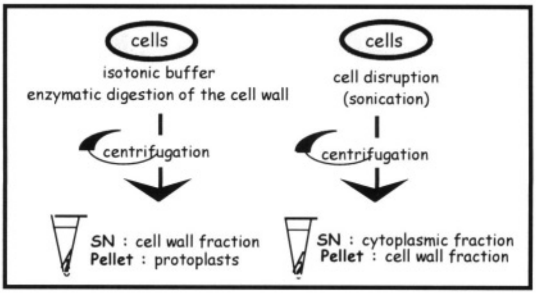

Fig. 1 - Standard procedures of cell fractionation for gram-positive bacteria. SN means supernatant.

In conclusion we believe that this method may open the possibility to study the hypothetical involvement of *Frankia* cell wall-associated proteins in the actinorhizal infection process.

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SEASONAL CHANGE OF THE ACTINORHIZAL NODULES AND THE MOVEMENT OF N IN *DISCARIA TRINERVIS*

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Discaria trinervis is a winter-deciduous actinorhizal shrub that grows in Northwest Patagonia, Argentina. The hyphae of *Frankia* penetrate the mature host cells of the nodule parenchyma and induce an increase in their size (hypertrophied cells) (Valverde, Wall, 1999; Berry, Sunell, 1990). Furthermore, the development of the symbiotic nodule tissue and the N_2-fixing activity correlate with the seasonal cycles in the host plants (Huss-Danell, 1997; Schwintzer et al, 1982). Our aim is to document the seasonal change of the actinorhizal nodules and the movement of N in *D. trinervis.*

The study area is a shrubland located in Pampa de Huenuleo (41°10' S, 71°21' W). The climate of this region is humid temperate, the N content of the soil is 0.2 mg g^{-1} dry soil, the P content is 4.4 μg g^{-1}, and the pH is 7.1. Young plants (about 1-3 year old) were collected each season. Nodules were separated and fixed in 4% glutaraldehyde. The roots, stems and leaves were dried and analysed for the N content (Kjeldahl method). The nodule lobes were excised in slices (25 μm) on a freezing microtome and stained with lactophenol cotton blue. The seasonal infection rate of nodules was estimated by determining the amount and size of hypertrophied infected cells (larger than 40 μm length) in the nodule lobes sections (16 replicates). The cell size was calculated by multiplying: cell width x cell length x π, (area of an ellipsis). The effectivity of this symbiosis was demonstrated by a nodulation assay with *D. trinervis* seedlings, inoculated with a crushed nodule suspension.

Seasonal behaviour was found for *D. trinervis* plants growing under natural conditions, with similar changes inside the symbiotic nodules to those described in *D. chacaye* and *Colletia hystrix* (Chaia, 1993). The higher N content of leaves in spring (2.7 % dry weight) and summer (2.5 % dry weight) coincided with the higher infection rate in nodules. It has been reported that the increments in temperature and day length induce the development of new photosynthetic tissue, renewed nodule cell division and the differentiation of vesicles (Wheeler, 1991). The N content of leaves dropped in autumn, before abscission, by 12% of the summer value, and a significant decrease in the number and size of hypertrophied cells was observed. (Kruskal Wallis test, H=11.482 and H=23.134, $p<0.05$). The lower infection rate found in autumn and winter fell upon with the higher content of N in roots (1.79 and 2.03% dry weight, respectively) and stems (2.19 and 2.07% dry weight, respectively). *Frankia* vesicles swelled from spring to autumn (2 to 6 μm) and were collapsed in winter. It seems that after the N reserves stored in root systems were consumed in spring for shoot outgrowth, new symbiotic tissue was produced for the N_2 fixing activity. This work was supported by Universidad Nacional del Comahue, grant 04/B705

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SECTION X

ENVIRONMENTAL STRESS

PHYSIOLOGICAL ROLES OF BETAINES IN *SINORHIZOBIUM MELILOTI* AND OTHER MEMBERS OF THE *RHIZOBIACEAE* SUBJECTED TO SALT STRESS

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1. Introduction

Sinorhizobium meliloti is able to survive osmotically stressful environments by the intracellular accumulation of compatible solutes, which restore the osmotic balance between the cell and the environment. Among these solutes, glycine betaine (the N-trimethyl derivative of glycine) has been shown to be a very efficient osmoprotective compound which strongly stimulates the growth rate of this bacterium in high-salt medium (Le Rudulier, Bernard, 1986). Unlike enteric bacteria, *S. meliloti* also catabolizes glycine betaine and uses it both as a carbon and a nitrogen source for growth. Glycine betaine either can be taken up directly from the environment by specific transport systems or synthesized from choline-O-sulfate or choline (Osteras et al., 1998; Smith et al., 1988). The osmoadaptative responses of other rhizobial species have not been thoroughly studied in comparison to those of *S. meliloti.* However, we have recently analyzed choline and glycine betaine uptake capacities and investigated the occurrence of glycine betaine biosynthesis pathway in various *Rhizobiaceae* strains which display a large variation in salt tolerance (Boncompagni et al., 1999).

2. Molecular analysis of glycine betaine biosynthesis in *S. meliloti* (*bet* genes)

In previous work, a *S. meliloti* Tn5 mutant (LTS 23-1020) lacking choline dehydrogenase activity was selected, and a cosmid clone was isolated by complementation from a pRK290 genomic library containing wild-type *S. meliloti* 102F34 DNA (Pocard et al., 1997). Molecular analysis of the *bet* locus identified four genes, *bet ICBA* organized in an operon. In addition to the *betBA* genes encoding, respectively, betaine aldehyde dehydrogenase (BADH), and choline dehydrogenase (CDH), the *betl* gene also shares homology to the *betI* gene of *E. coli.* Its product is likely to be involved in the regulation of the *S. meliloti bet* operon. The most striking finding was the presence of novel gene *betC,* located between *betI* and *betB* (Osteras et al., 1998). The BetC protein share significant homology to sulfatases and was identified as a choline sulfatase by enzymatic assays. To demonstrate that choline-O-sulfate is converted into choline, cell extracts were incubated with ^{14}C-choline-O-sulfate, and the production of ^{14}C-choline was monitored. With wild-type cell-extracts, ^{14}C-choline was produced only when the cells were grown in presence of choline-O-sulfate or choline. The presence of 0.3M NaCl in the growth medium slightly reduced the choline sulfatase activity. No ^{14}C-choline was produced with extracts obtained from a mutant strain *betC*::Tn*5* grown in presence of choline-O-sulfate, clearly indicating that the choline sulfatase activity is encoded by the *betC* gene. The substrate specificity of the enzyme was analyzed by determining the ability of different choline esters to compete with choline-O-sulfate as substrate : only phosphorylcholine showed significant competition.

In order to identify the factors affecting the regulation of the *bet* locus and define the relative importance of the osmotic and nutritional roles of glycine betaine, transcriptional *lacZ*-fusions of the

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bet A and *betB* genes were constructed and recombined into the genome of *S. meliloti* 2011. In cells grown *in vitro,* the expression of *betA* and *betB* was strongly induced by the presence of choline or choline-O-sulfate, independently of the osmotic pressure or the presence of other carbon or nitrogen sources. The expression of the transcriptional *bet* fusions was also detected *in planta* during the interaction with the plant host (*Medicago sativa*). Both genes were transcribed at all stages of the nodule development, from the initiation of the infection thread to the mature nodules. These results suggest that choline and/or choline-O-sulfate are available for the free-living bacterium and the bacteroid.

3. Occurrence of choline and glycine betaine uptake and metabolism in the family *Rhizobiaceae*

Although the choline-glycine betaine pathway has been well characterized in *S. meliloti,* the occurrence of this pathway was poorly known in other members of the family *Rhizobiaceae.* Therefore, we have analyzed choline and glycine betaine uptake capacities, and measured BADH and CDH activities in various *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium* and *Agrobacterium* reference strains which display a large variation in salt tolerance. When provided in a medium of elevated osmolarity, choline and glycine betaine enhanced the growth of *R. tropici, S. meliloti, S. fredii, R. galegae, A. tumefaciens, M. loti,* and *M. huakuii,* demonstrating their utilization as osmoprotectants. However, both compounds were inefficient for the most salt-sensitive strains, such as *R. leguminosarum* (all biovars), *A. rhizogenes, R. etli,* and *B. japonicum.* All strains, except *B. japonicum,* exhibit transport activity for choline and glycine betaine. When the medium osmolarity was raised, high-affinity choline uptake activity was inhibited, whereas glycine betaine uptake was either increased in *R. leguminosarum* and *S. meliloti* or, more surprisingly, reduced in *R. tropici, S. fredii,* and *M. loti.* BADH and CDH activities were present in all strains with the exception of *M. huakuii* and *B. japonicum.* The enzymatic data were confirmed by Southern analysis of genomic DNAs from the different rhizobial species using *betA*- and *betB*-specific probes from *S. meliloti* : the presence of a single homologous gene was detected in all tested strains, except in *M. huakuii* and *B. japonicum.*

With the exception of *B. japonicum,* all strains were able to use glycine betaine and choline as sole carbon and nitrogen sources (Boncompagni et al. 1999). This catabolic function, reported for only a few soil bacteria, could increase competitiveness of rhizobial species in the rhizophere. The main physiological role of glycine betaine in the family *Rhizobiaceae* seems to be as an energy source, while its contribution to osmoprotection is restricted to certain strains.

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5. Acknowledgements

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GENETIC CIRCUITS INVOLVED IN THE RESPONSE OF ROOT NODULE BACTERIA TO LOW pH

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The *Sinorhizobium*-legume interaction is sensitive to a number of environmental factors; one of the important stresses is soil acidity. Low pH imposes severe constraints on legume productivity. In the *Sinorhizobium -Medicago* partnership, the prokaryotic partner is the most acid-sensitive. The isolation of acid-tolerant *Sinorhizobium* strains from the eastern and southern Mediterranean rim have allowed the successful development of medic pastures on over 1 million ha of land in Western Australia, posing two important questions:

- How do acid-tolerant strains differ from acid-sensitive counterparts?
- How do agriculturally important root nodule bacteria respond to low pH?

Knowledge of the genetic circuits required and activated by the cell in acidic conditions is vital to understand the response and survival of bacteria in these conditions. The molecular basis of acid tolerance has been investigated with several approaches (Glenn et al., 1999).

1. Insertional mutagenesis

Construction of acid-sensitive mutants of *S. meliloti* has identified genes essential for acid tolerance (Tiwari et al., 1996a, Tiwari et al., 1996b). These mutants show pleiotropic phenotypic defects (Table 1)..

Table 1. Low pH sensitive mutants isolated by insertional inactivation

Mutant	Calcium response	known sensitivity	Lesion	Function
Sm TG2-6	Y	Low pH, Zn^2 Cu^{2+}, Azide	*actA*::Tn*5*	Lipid metabolism
Sm TG5-46	Y	Low pH, Zn^{2+}, Azide	*actR*::Tn*5*	Regulator
Sm RT295	Y	Low pH, Zn^{2+} Azide	*actS*:*Ω*Km	Sensor
Sm RT3-27	N	Low pH, Cu^{2+}	*actP*::Tn*5*	P-type ATPase
Rl WR1-14	N	Low pH, Cu^{2+}	*actP*::Tn*5*	P-type ATPase
Sm WRR1 (3-27R1)	Y	Low pH, Zn^{2+}	*exoH*::Tn*5*	Succinylation

Sm, S*inorhizobium meliloti*; Rl, *Rhizobium leguminosarum* bv *viciae*.

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Protein products critical for cell survival at low pH include: ActA, apolipoprotein acyl transferase; ActR, a response regulator; ActS, a sensor and ActP, a CPx heavy metal transporting ATPase

2. Proteomic analysis

Two dimensional gel electrophoresis was used to analyze the total protein complement of cells grown in the absence and presence of a persistent and transiently applied acid stress (Reeve et al., 1998a). We found that a number of proteins were either persistently up or down regulated by acid stress or transiently up or down regulated directly after an acid stress was applied. N-terminal sequence analysis has identified two of the transiently down regulated proteins to be GlnK (regulator of GlnA) and GlnA (glutamine synthetase), suggesting a link between nitrogen metabolism and pH stress. Two proteins could not be sequenced due to amino acid modifications and the remaining nine sequenced proteins could not be identified by database searches.

3. Transcriptional *gusA* fusions.

Cassettes and minitransposons have been constructed with and without a promoterless *gusA* reporter gene and contain the gene conferring resistance to kanamycin (*nptII*) or gentamicin (*aacCI*) (Reeve et al., 1999). These constructs were used to generate low pH responsive transcriptional fusions. Cloning and sequencing rhizobial DNA flanking the fusion has identified the *genes phrR* (Reeve et al., 1998b) and *lpiA* (Reeve et al., 1999). Although these genes are up-regulated by low pH, they are not essential for growth in acid media. The promoter for *lpiA* is responsive specifically to low pH while *phrR* is regulated by a variety of stresses.

Transcriptional fusions were also created in an *actS* mutant background to identify ActS/R-responsive The protein products of the genes regulated by this two-component system are involved in diverse physiological functions.

At present, three independent regulatory circuits have been identified. The ActR/S circuit is essential for growth at low pH. This circuit does not regulate the expression of either *lpiA* or *phrR*. The second circuit is regulated by numerous stresses (including low pH) through the activation of the PhrR regulator. The third circuit is specific to acidic pH and involves the up-regulation of *lpiA* by an unidentified regulator. The current model for the response of *Sinorhizobium* to acidity will be presented.

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PERSISTENCE AND COMPETITIVENESS OF OR191-LIKE RHIZOBIA AND *SINORHIZOBIUM MELILOTI* (*SME*) STRAINS IN AN ACIDIC SOIL OF ARGENTINA.

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1. Introduction.

Acid soils represent a severe constraint for alfalfa crop in Argentina and Uruguay. The nitrogen-fixing symbiosis established between *S. meliloti* and alfalfa becomes seriously altered in acid environments accounting for a significant reduction in the yield and persistence of cultures. In our laboratory we are investigating the basis of symbiosis development at low pH, and the mechanisms of acid tolerance in alfalfa nodulating rhizobia (ANR).

We have previously reported the isolation of a competitive and efficient strain, *S. meliloti* LPU63, from a collection of more than 400 local isolates (Segundo et al, 1998). Within the same collection of ANR we also detected the presence of a rare type of AT, inefficient, and competitive rhizobia related to the previously described strain *R.* Spp. Or191 (Del Papa, et al., 1999).

To characterize the interaction of S. *meliloti* LPU63, Or191-like isolates, and native rhizobia under natural acidic conditions, we evaluated their persistence, and competitiveness in the open acid field.

2. Material and Methods

2.1 Nodulation assays in the laboratory

Soil samples from the acidic field were in the laboratory to evaluate the bacterial competitiveness against native and co-inoculated rhizobia.
Non-sterile Soil samples (pH 5.6) were inoculated with approx. 106 u.f.c./g soil of S. meliloti LPU63, S. meliloti 2011, and R. Spp LPU83.

2.2 Field release experiments

The experiment was designed to include 3 different rhizobia: *S. meliloti* LPU63 and 2011, and *R.* Spp. LPU83. The experiment was carried out in a field of with the following characteristics: soil pH 5.6, organic matter 2.67%, organic C 1.55%, total N 0.15, C/N ratio 10.3, assimilable phosphorus 15.4 ppm (INTA Castelar, Province of Buenos Aires, Argentina). A total of 24 parcels of 1.2 x 2 m distributed in random blocks were designed (4 parcels corresponded to the control soil without inoculation). Single and double inoculations were performed. The number of ANR in the control soil was found to be approx. 3 x 102 rhizobia/g of soil, as estimated by the method of the most-probably-number (MPN).

3. Results and discussion

Single and double inoculations were performed in the field to compare the behavior of strains LPU63 and LPU83 previously characterized in hydroponic media (Segundo et al., 1998). Soil samples from the acidic field were used in a parallel experiment under laboratory conditions using plastic pots. Root nodules in the acidic field appeared late as expected, between the second and the third month post-inoculation. A higher competitiveness of strain LPU83 against the native rhizobia was observed. However, nodulation of this bacteria was very poor in the primary root (Figure). This was in agreement with previous results obtained in hydroponic media. Nodulation tests in pots paralleled well the nodule occupancy observed in the field. Laboratory containment

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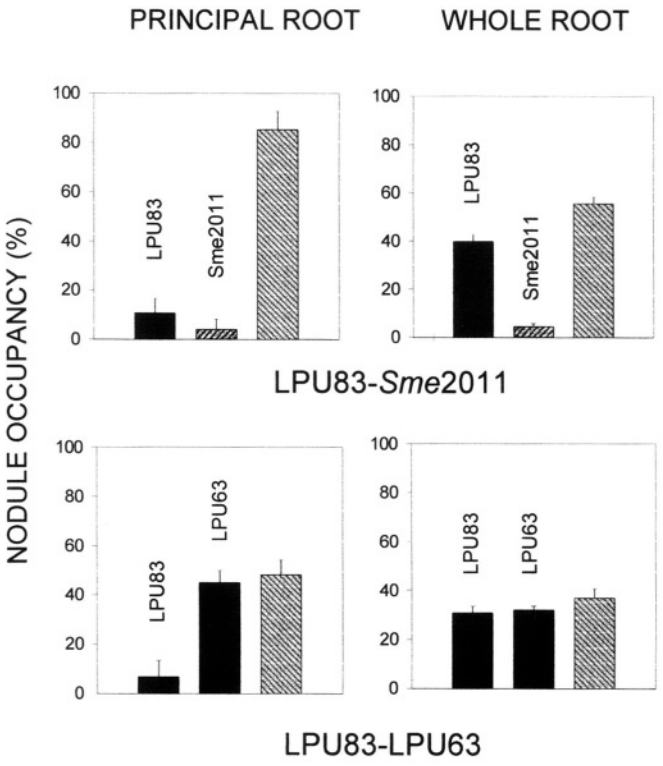
systems using non-sterile soil was found to be a reliable system for evaluation of strains under acid-stress.

Interestingly, the efficient strain *S. meliloti* LPU63 resulted a good competitor for nodule occupancy in the field against the acid tolerant and inefficient *R.* spp. LPU83, and also against the native population. It is the important observation that the proportion of nodule occupancy of strain LPU63 regarding LPU83 was significantly higher in primary roots, suggesting that the shinorhizobia developed the earlier infections on the tap root. The step-by-step evaluation of strain LPU63 (hydroponyc media-soil samples-open field) showed this rhizobia as a suitable strain to be evaluated in more extended assays in agricultural acidic fields.

Figure: Nodule occupancy of a coinoculation assays in an acid field (pH 5.6) of Central Argentina.



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A REQUIREMENT FOR THE IRON-REGULATED OUTER MEMBRANE PROTEIN FEGA IN THE *BRADYRHIZOBIUM JAPONICUM/SOYBEAN* SYMBIOSIS

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One of the main goals of rhizobial research is to understand how the bacterial symbiont interacts with the host plant to regulate metabolic processes essential for the nitrogen-fixing symbiosis. Our own research is focused on iron in the symbioses. Iron-containing proteins figure prominently in the nitrogen-fixing symbioses between rhizobia and their respective plant hosts (Guerinot 1993). Iron acquisition in the soil can be problematic due to the low solubility of ferric iron [Fe(III)] in aerobic environments at neutral pH (Guerinot and Yi 1994). Therefore, as free-living soil microorganisms, rhizobia must have a way to solubilize iron as well as a way to compete for this nutrient with other organisms present in the rhizosphere. As endosymbionts, rhizobia must have mechanisms for acquiring iron from the surrounding plant tissue. Many rhizobial and bradyrhizobial strains are able to produce siderophores under iron-deficient growth conditions. Siderophores are small molecular weight compounds with a high affinity for Fe(III). Siderophores are synthesized and secreted by microorganisms and fungi under conditions of iron limitation (Guerinot 1994; Neilands 1995). Most siderophores can be classified in two groups: hydroxamate siderophores, mostly produced by bacteria and fungi and phenolate-catecholate siderophores mostly produced and used by bacteria. Siderophore levels can be as high as 10 μM in the rhizosphere (Crowley et al. 1987). Many bacteria can utilize siderophores produced by other organisms (Buyer and Sikora 1990). In order to utilize a siderophore, an organism must have a siderophore-speciflc iron uptake system composed of four main components: a high-affinity outer membrane receptor, the inner membrane-anchored TonB protein, a periplasmic binding protein and several inner membrane-associated proteins (Braun 1995). The genes encoding these proteins are coordinately derepressed under conditions of iron deficiency and are negatively regulated by the product of the *fur* (ferric uptake regulation) gene (Guerinot 1994).

Are siderophores produced *in planta*? It is important to point out that we currently have no direct evidence that bacterial siderophores are produced *in planta* in any of the rhizobial/legume symbioses. However, there is one report of siderophore-bound iron in the peribacteroid space of soybean nodules (Wittenberg et al. 1996). Data obtained in our laboratory indicate that iron may indeed be stored in the peribacteroid space (Levier and Guerinot 1996).

In cultured cells of *B. japonicum,* certain outer membrane proteins are only made when cells are grown under iron deficiency; these are presumably involved in iron uptake. Three main iron-regulated outer membrane proteins from *B. japonicum* strain 61A152 have been characterized. Our goal was to clone the genes which encode these iron-regulated outer membrane proteins, then create gene knock-outs of the corresponding genes and characterize the mutants with respect to iron uptake, both in culture and *in planta.* We had previously used degenerate primers, designed on the basis of protein microsequence from one of these proteins, to isolate a gene, *fegA* (Levier and Guerinot 1996). *fegA* encodes a protein which shows significant similarity to members of the hydroxamate family of siderophore receptors. The transcriptional start site of *fegA* was mapped by primer extension analysis and a putative Fur-binding site was found in the promoter. Primer extension and RNA slot blot analysis demonstrated that *fegA* was only expressed in cells under iron-limiting conditions. We have previously shown that *B. japonicum* is able to use a variety of fungal siderophores as iron sources, including the hydroxamates ferrichrome and rhodotorulic acid (Plessner 1993).

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We have constructed *a fegA* chromosomal mutant strain. This mutant strain shows no ability to synthesize FegA under iron deficient growth conditions. We checked the ability of bacteria to grow on iron limited agar medium (1mM EDDHA iron chelator) when supplied with various siderophores (Noya et al. 1997). Under these growth conditions, the mutant strain cannot use ferrichrome or rhodotorulic acid whereas the wild type strain is fully able to use these compounds. Meanwhile, others siderophores, like citrate, hemin and ferrioxamine, can be used by both strains. In order to determine whether the *B. japonicum* FegA protein plays an important role in the symbiosis, we inoculated soybeans with the *fegA* mutant and found a dramatic phenotype *in planta.* Four weeks after inoculation, nodules do not contain leghemoglobin and are nearly unable to fix nitrogen. Nodules formed by the *fegA* mutant are smaller than those formed by wild type. A delay in nodule formation is also noticeable. Plants inoculated with the mutant strain have a lower shoot dry weight than plants inoculated with the wild type strain. The analysis of the mutant phenotype was investigated further using electron microscopy. No difference could be seen at the level of the infection thread. But nodules formed by mutant strain have very low number of symbiosomes and contain numerous vesicles. Vesicles are shaped like peribacteroid units but lack any bacteroids inside. These symbiosomes and vesicles seem to be quite stable with no sign of early degradation and they persist through week 6. Most of the symbiosomes formed by the *fegA* mutant have a large peribacteroid space with only one bacteroid. Similar vesicle-filled nodules have been previously described for several random Tn5 insertion mutants of *B. japonicum* (Regensberger et al. 1986; Roth and Stacey 1989).

The dramatic symbiotic phenotype suggests that FegA is an essential component of the symbiotic iron transport system that the bacterium employs to acquire iron *in planta.* Further biochemical investigation of FegA may illuminate the nature of the iron compounds provided *in planta.*

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2D SDS PAGE OF PROTEINS INVOLVED IN ACID TOLERANCE IN *RHIZOBIUM TROPICI* CIAT 899

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Our aim was to detect proteins which mediate acid tolerance mechanisms in rhizobia. The different responses of rhizobia to acidity are mediated by at least 20 different genes (Glenn, Dilworth, 1994). Genes involved in sensing (ActS) and regulating (ActR) growth under acidic conditions have been identified through analysis of acid sensitive Tn5 mutants from *Rhizobium meliloti* (Tiwari et al., 1996a) as well as ActA which is involved in sensitivity to acidity and copper (Tiwari et al., 1996b). Naturally occurring acid tolerant rhizobial strains are very useful for investigating acid tolerance mechanisms. *R. tropici* CIAT899^T is an acid tolerant strain capable of nodulation at pH 4.0 (Graham et al., 1994). It accumulates glutamate and glucose under acid stress (Graham et al., 1994). The presence of a carbon source greatly improves rhizobial survival under acidic conditions (Clarke et al., 1993) and the ability to take up glucose after pH shift differs between acid tolerant *R. tropici* CIAT899^T and acid sensitive CFN42, Kim5S and *R. etli* CIAT611 (Steele et al., 1999). *R. tropici* CIAT899^T is highly competitive under acidic conditions (Streit et al., 1992). *R. tropici* CIAT899^T pumps out calcium as a response to pH shift (Aarons, Graham, 1991). An investigation of protein expression before and after pH shift found that 5 proteins were up-regulated and 4 proteins down-regulated after pH shift, and no new proteins were detected (Peick et al., 1999). To investigate mechanisms of acid tolerance, acid sensitive Tn5 insertion mutants were created from the acid tolerant strain *Rhizobium tropici* CIAT899^T (Vinuesa, 1998). *R. tropici* CIAT899^T mutants were replica plated onto minimal medium (MM) at pH 6.8 to check for auxotrophs, on minimal acidified medium (MAM) plates at pH 4.2 to check for acid sensitivity, and MAIM plates (with 250 μM $AlCl_3.6H_2O$) to check for sensitivity to aluminium toxicity at pH 4.7. Acid sensitive mutants were selected as those that grew on MM but showed reduced or no growth at all on MAM plates. Southern blot hybridisation analysis of the acid sensitive mutants with a Tn5 specific probe indicated that all mutants have a single transposon insertion at different locations. All mutants have reduced competitiveness and nodulation ability (10% of wild-type) and all form Fix$^-$ nodules. Sequencing of the region flanking the Tn5 insertion of mutant PV9 indicated the disrupted gene was homologous to a Ca^{2+} channel protein (Vinuesa, 1998).

The presence of calcium enables *Sinorhizobium meliloti* and *Rhizobium trifolii* to grow under acidic conditions (Reeve et al., 1993: Watkin et al., 1997). Although the role of calcium in rhizobial growth under acidic conditions remains unclear, calcium may be involved in maintenance of the cell membrane or in regulation of internal pH (Ballen et al., 1998). *Rhizobium tropici* CIAT899 pumps out calcium as a response to pH shift (Aarons, Graham, 1991). Differences in calcium repairability have been detected in acid sensitive Tn5 insertion mutants (Tiwari et al., 1992). We tested *R. tropici* CIAT899 wild-type and acid sensitive mutants PV3, PV4 and PV9 for calcium repairability. Cultures were grown to $OD_{600} = 0.4$ in defined minimal medium at pH 6.8 with 3 mM calcium. They were then divided into 3 aliquots, centrifuged, washed and resuspended. The control was resuspended in medium at pH 6.8 with 3 mM Ca; the second in minimal medium at pH 4; and the final aliquot at pH 4 with 10 mM Ca. The growth of the cultures was followed with OD_{600} and cfu/ml, and the pH of the medium was measured at the start and end of the experiment. The wild-type grew slightly more slowly at pH 4 and showed no improvement in growth at pH 4 with calcium. Mutant PV3 grew more slowly at pH 4 and growth was greatly improved by the presence

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of calcium. Mutant PV9 was more sensitive to acidity than PV3 and it was not repairable. PV9 was the mutant which appeared to be mutated in a Ca^{2+} channel protein. PV4 was the most sensitive mutant and did not grow at all at pH 4 even with calcium.

To detect proteins which are differentially expressed under acidic conditions, mutants PV3, PV4 and PV9, and *R. tropici* CIAT899 wild type were grown at pH 6.8 and pH 5.5 (buffered with 20 mM MES) and also shifted from pH 6.8 to 5.2 for 45 min. The proteins were extracted and separated by 2D SDS PAGE using IPGs [pH 4 - 8] in the first dimension and 14% acrylamide in the second dimension. The protein patterns were compared and 12 differentially expressed proteins identified. Amino acid sequences were obtained for 9 of them. Some proteins were expressed similarly by both mutants and wild-type after pH shift and they represent acid response mechanisms which remain intact within the mutants. The rest of the proteins are expressed differentially. These proteins may be directly or indirectly influenced by the mutation. One protein from mutant PV9, which is expressed at a lower level than the wild-type after pH shift, produced a homology (79% identity in 24 aa overlap) with an amino acid binding protein (Aapj) from *R. leguminosarum* (Walshaw, Poole, 1996). This protein is involved with the efflux of solutes and uptake of amino acids such as glutamate, which is accumulated by *R. tropici* CIAT899 under acid stress (Graham, et al., 1994). One protein missing from PV4 has homology to a NIFR3-like protein (83%) from *R. leguminosarum* bv. *phaseoli.* Differentially expressed proteins from PV4 and PV3 produced homologies to a transcriptional elongation factor from *Rhizobium leguminosarum* and a nucleoside diphosphate kinase (Ndk), respectively. It is difficult to speculate about the roles of these proteins in acid tolerance without having first shown that they are differentially expressed as a result of increased acid sensitivity. Therefore, it was important to find the coding genes for the proteins.

Primers were designed from the amino acid sequences of Aapj and Ndk and used in PCR to obtain the coding gene. A product of the expected size (900 bp) was obtained with the Aapj primers, which was ligated into a pGEM-T vector and sequenced. Its nucleotide sequence was compared with the database and showed the highest homology with Aapj from *R. leguminosarum.* This plasmid and insert have been digested with Sal1, resulting in 3.4 kb and 550 bp fragments. The larger fragment was self ligated and the smaller fragment was cloned into a mobilizable vector. The next step is to use this in mutagenesis of the wild-type and test the resultant mutants for their acid sensitivity.

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A MUTATION IN THE *GUA*B GENE OF *R. TROPICI* AFFECTS THERMAL TOLERANCE AND BEAN NODULATION

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1. Introduction

Soil bacteria are often exposed to environmental constraints such as nutrient limitations, varations in pH and temperatures shifts. The ability of rhizobia to persist under high thermal conditions seems to be a prerequisite allowing colonization and subsequently nodule formation. High soil temperature in tropical areas is indicated to be a major problem for biological nitrogen fixation by common beans (*Phaseolus vulgaris* L.), and variability in thermal tolerance in bean nodulating rhizobia had been shown. Hungria et al. had examined a collection of isolates from Brazil and identified several bean nodulating strains able to nodulate beans and fix nitrogen under conditions of high temperature (Hungria et al., 1993). We have examined a Tn*5-luxAB* induced *R.tropici* CIAT899 mutant (CIAT899-10T) that is pleiotropically affected in its intrinsic thermal tolerance and as it was also previously described for purine auxotrophic rhizobium strains, CIAT899-10T fails to effectively nodulate bean plant.

2. Material and Methods

Strain CIAT899-10T is a Tn*5-luxAB* mutant derived of wild type strain *Rhizobium tropici* CIAT899. Rhizobia cells were grown in TY medium or minimal medium GTS. Sequence determination was done by using Sequenase kits (US Biochemicals).

3. Results and Discussion

Strain CIAT899-10T was found to grow at a slower rate as compared to the parent strain in liquid media at 28 °C, with a mean generation time of ~ 5 hours, but was found to be unable to grow when temperature was shifted to 40°C. To characterize the gene mutated with the Tn*5-luxAB* in strain CIAT899-10.T, the tagged locus was cloned from the rhizobial genome. This was facilitated by the presence of an origin of replication within the Tn*5-luxAB* transposon (Wolk et al., 1991). Total genomic DNA of strain CIAT899-10T was digested with restriction enzyme EcoRI, selfligated and transferred into *E.coli.* The DNA sequence of the *R. tropici* fragments adjacent to the transposon-DNA was determined by using unique primers corresponding to both ends of the Tn*5-luxAB* and also appropriate primers that were deduced of the resulting DNA sequence data. Sequence analysis revealed an open reading frame of 1485 nucleotides showing an ATG start codon preceded by a putative ribosomal binding site. The predicted protein deduced of this ORF shares 60% identical residues with the *gua*B gene product *of E.coli* and of several other bacteria. The *gua*B encodes the enzyme inosine monophosphate (IMP) dehydrogenase which catalyzes the conversion of inosine 5'-monophosphate into xanthosine 5'– monophosphate in the guanosine biosynthetic pathway.

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However, the strain CIAT899-10T is not an auxotrophic mutant for guanine due to an alternative pathway via xanthine dehydrogenase that provides precursors for guanine production.

We investigated the induction of heat shock proteins (HSP) in the strain CIAT899-10T as follows. Rhizobial cells were incubated in the presence of ^{35}S-methionine and samples were removed before and one 15 minutes after shifted from 28 °C to 45 °C. Samples were subjected to SDS-PAGE and proteins revealed by autoradiography. It was found that the wild type strain and strain CIAT899-10T have the same pattern of heat induced proteins (Figure 1). Assuming that ppGpp communicates stress signals to the thermal stress response genes and that in the *guaB* mutant the availability of GMP for ppGpp synthesis may result limited, we quantified levels of ppGpp in wild type strain and mutant strain CIAT899-10T. ppGpp was determined by HPLC after formic acid extraction. Howorth and England had described *R. tropici* CIAT899 to behave as a relaxed strain (Howorth and England, 1999). In our studies we found that wild type strain and mutant do not accumulate ppGpp following thermal shock at 50 °C indicating that in order to initiate responses that permit *R. tropici* to grow at high temperature, ppGpp is not required.

The appearance of bean nodules elicited by the mutant strain was very similar to normal nodules but they were smaller and white in colour. Microscopic observation revealed clear differences between wild type and mutant nodules. Wild type-induced nodules exhibited infection threads and the central zone with rhizobia infected cells whereas no infection threads nor rhizobia released within the cells were detected in the mutant nodules. Bacteria were observed in the intercellular space. Cells were vacuolated with large starch grains. This symbiotic phenotype seems to be similar to that found with mutants in *pur* genes coding functions for earlier steps of the biosynthetic pathway for purines (Newman et al., 1995).

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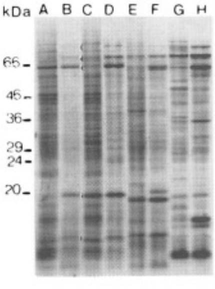

Figure 1. Heat shock proteins in Rhizobia strains. Proteins were separeted by SDS-PAGE in 12% gel. Arrow-heads indicate the hsp in *R. tropici* CIAT 899. Molecular mass standars are indicated on the left margin.
Lanes: A, *R. tropici* CIAT 899, 28°C; B, *R. tropici* CIAT 899, 45°C; C, *R. tropici* CIAT 899-10.T, 28°C; D, *R. tropici* CIAT 899-10.T, 45°C; E, *S. meliloti* 1021, 28°C; F, *S. meliloti* 1021, 45°C; G, *E. coli* DH5□, 37°C; H, *E. coli* DH5α, 45°C.

A NEW PUTATIVE SIGNALLING CIRCUIT INVOLVED IN THE REGULATION OF CELLULOSE SYNTHESIS IN *RHIZOBIUM* AND *AGROBACTERIUM*.

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Members of the genera *Rhizobium* and *Agrobacterium* are known to produce cellulose fibers during early stages of the infection process. We have identified two tandemly located putative response regulators *celR1* and *celR2,* involved in the regulation of cellulose synthesis in *R. leguminosarum* bv. *trifolii.* We observed a gene dosage dependent activation of cellulose synthesis in wt *R. trifolii* but also in *A. tumefaciens,* upon introduction of a plasmid carrying *celR1celR2* into these bacteria. This indicates that the induction pathway might be similar in these organisms. A BLAST search revealed a number of proteins from different organisms with a conserved sequence motif GGDEF in their C-terminus, exhibiting sequence similarity to CelR2. The closest homolog to CelR2 is the PleD protein from *Caulobacter.* A gene, *yhcK,* encoding a protein with a GGDEF motif in *E. coli,* was cloned in a plasmid and introduced into a *celR2*-defective mutant and into wt *R. trifolii* and *A. tumefaciens.* Interestingly, this gene complemented the *celR2* defect and caused increased production of cellulose in wildtypes of both species. The biological significance of the compatibility of these regulators is under investigation. Experiments are being performed to identify and characterize other components of the putative regulatory circuit in these organisms.

DOES PHOSPHORUS DEFICIENCY AFFECT THE SPECIFIC EXPRESSION OF CARBONIC ANHYDRASE GENE IN THE *PHASEOLUS VULGARIS* NODULE PARENCHYMA ?

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1. Introduction

In the *Rhizobium*-legume symbiosis, nitrogen fixation is limited by intranodular oxygen, controlled by nodule conductance to O_2 diffusion (g_{no}). The g_{no} is altered by environmental limitations of symbiotic N_2 fixation such as phosphorus deficiency (Ribet, Drevon, 1995), and is presumably regulated by osmocontractile cells of the nodule parenchyma surrounding the infected N_2-fixing zone (Drevon et al., 1997). A nodule carbonic anhydrase (CA) gene expresses in the parenchyma of alfalfa nodule (De La Pena et al., 1997).

To address whether the nodule-specific CA is involved in the osmoregulation of nodule O_2 diffusion, we have (i) examined the exact localization of CA transcripts in common bean nodule parenchyma, and (ii) tested whether the intensity of the nodule CA gene expression is related to the effect of phosphorus deficiency on the nodule O_2 permeability.

2. Methodologies

Phaseolus vulgaris (BAT477) was inoculated with *Rhizobium tropici* (CIAT899), and grown in aerated-hydroponic culture with weekly supply of 250 (P-sufficient) versus 75 (P-deficient) µmol P. Nodule conductance to O_2 was measured on intact plant according to Ribet et al. (1995). Nodule cell morphometry was measured by image analysis like in Serraj et al. (1995).
In situ hybridization was performed with a radiolabeled *PvCA* probe on serial cuts of nodules after embedding in a methacrylic resin.

3. Results

The CA expression was restricted to 2-4 cells-layers, defined as inner-cortex (IC) cells. These IC-cells were localized between the vascular traces and the distributing zone (most internal non-infected cells surrounding the infected zone) within nodule parenchyma. The phosphorus deficiency increased the amount of CA transcripts. The nodule conductance to O_2 and IC-cell size were also increased, though the nodule mass and the plant N_2-dependent growth were decreased.
Thus, CA may interact with nodule permeability, though the relation might be indirect.

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THE *EXOH* GENE AND ACID TOLERANCE IN *SINORHIZOBIUM MELILOTI*

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1. Introduction

The establishment of the *Rhizobium*-legume symbiosis is affected by soil acidity due to the sensitivity of the prokaryotic symbiont (Munns, 1986). Exopolysaccharide (EPS), carbohydrate polymers secreted by a variety of bacteria, play an important role in the interaction of root nodule bacteria promoting nodule development and invasion of legumes *Rhizobium* species. A protective role for EPS has been suggested in low pH tolerance of rhizobial strains (Cunningham, Munns, 1984). 327-R1, an acid-sensitive mutant of *S. meliloti* strain WSM 419, was isolated by Tn5 mutagenesis. This mutant has a unique Tn5 insertion in *exoH,* a gene required for the succinylation of the acidic EPS succinoglycan (EPSI). 327-R1 is of particular interest as it produces a non-succinylated EPS and shows a very acid-sensitive and zinc-sensitive phenotype. The aim of this study is to explore the connection between *exoH and* acid tolerance.

2. Results and Discussion

pEX154, a plasmid carrying 18 Kb of DNA including the *exoH-exoP* cluster from *Rhizobium meliloti* RM1021 (Leigh et al, 1987), restores the succinyl modification to the EPS and can complement the low pH sensitive and zinc-sensitive defect of 327-R1. To localize the gene(s) responsible for complementation, pEX154 was restriction digested and subclones spanning different regions were constructed. These subclones were mobilized into the mutant 327-R1 and phenotypes were studied by spotting 10^4 cells on appropriate media. The plasmid pAV-lC, an 11.5Kb DNA fragment obtained by *Hind*III digestion and religation of pEX154, also showed the same responses as pEX154. To obtain a smaller fragment containing the *exoH* gene, the plasmid pAV-lC was subcloned to construct pAV-19 and pAV-446. Both these clones failed to complement the pH-sensitivity defect of 327-R1. The plasmid pAV-19 partially restore EPS succinylation shown by its ^{1}H-NMR spectra suggesting that either restoration of succinylation is not enough to complement low pH sensitive defect, or the *exoH* gene by itself does not have a direct role in growth at low pH. The plasmid pAV-582 was constructed carrying the *exoH* gene on a 5.1 Kb DNA fragment. 327-R1 containing pAV-582 fluoresced brightly on calcofluor-supplemented LB plates, confirming a restoration of the succinyl modification of EPS, but still failed to grow on JMM minimal medium (pH 5.6). This result suggests that the *exoH* gene may not have a direct involvement in acid tolerance. The plasmids pAV-483 and pAV-2J were constructed to study the possibility of other *exo* genes in the vicinity of *exoH to* be involved in acid tolerance. Both of these plasmids failed to complement the low pH sensitivity defect of 327-R1. The minimun genetic requirement for restoration of acid- and zinc-tolerance is on the plasmid pAV-lC; smaller clones do not restore these defects and a combination of genes may therefore be required.

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ACTR IS A GLOBAL GENETIC REGULATOR IN *SINORHIZOBIUM MELILOTI*

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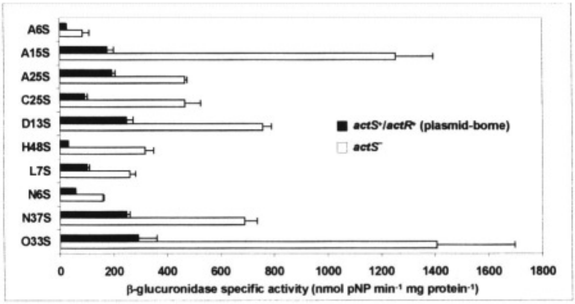
1. Introduction

Previously we have described a two-component signal transduction system comprised of ActS (histidine kinase) and ActR (response regulator) in *Sinorhizobium meliloti* WSM419. This study focuses on identifying the genes that are transcriptionally regulated by the ActS/R system.

2. Results & Discussion

Construction of transcriptional fusions. It was reasoned that if a gene was responsive to ActS/R it would show a difference in expression between wild-type and mutant genetic backgrounds. To test this hypothesis an *actS*:ΩSm mutant of *S. meliloti* WSM419 was mutagenised with mTn5-GNm (containing a promoter-less *gusA* gene) to create a library of *gusA* transcriptional fusions. β-Glucuronidase (GUS) activity was quantified in an *actS*$^-$ and *actS*$^+$/*actR*$^+$ (plasmid-borne) background for each mutant. Mutants showing significant differences in GUS activity between the two genetic backgrounds were selected for further study.



Sequence analysis. Sequences obtained from DNA surrounding the ActS/R-responsive fusions were compared to database submissions using the BLASTX programme. Sequence analysis identified a complete *cbb* operon located on a 30 kb insert cloned from the mutant A15S. This operon encodes enzymes required for CO_2 assimilation via the Calvin-Benson-Bassham (CBB) pathway.

Mutant	Gene similarity & function		Significance of similarity
A6S	*glgP*	Glycogen phosphorylase	3.1×10^{-75}
A15S	*cbbS$_l$*	RuBisCO small subunit	6.8×10^{-51}
A25S	*treY*	Trehalose synthase	1.6×10^{-67}
C25S	*fdsA*	Formate dehydrogenase α-subunit	7.6×10^{-17}
D13S	NS		
H48S	*orf2*	Sugar permease	5.4×10^{-14}
L7S	ND		
N6S	*agpA*	α-Galactoside binding protein	1.3×10^{-72}
N37S	*bphX0*	Glutathione *S*-transferase	4.8×10^{-12}
O33S	ND		

ND. not determined: NS. no significant similarity.

Regulation of C_1 metabolism. Activities of formate dehydrogenase (FDH), formaldehyde dehydrogenase (FALDH) and methanol dehydrogenase (MDH) were measured using wild-type, *actS*:ΩSm and *actR*::Tn*5* cell extracts. Inactivation of either *actS,* or *actR,* resulted in a significant increase in C_1 metabolic enzyme activity, suggesting that ActS/R function as regulators of C_1 metabolism in *S. meliloti.* Derepression of *cbbS*::*gusA* occurred in an *actS*$^-$ background, whereas expression was significantly lower in an *actR*$^-$ background compared to wild-type cells. This indicates that ActR functions as a positive regulator of the *cbb* operon of *S. meliloti.*

A GENE REGION CONFERRING STRESS TOLERANCE TO *RHIZOBIUM LEGUMINOSARUM BV VICIAE* AND *SINORHIZOBIUM MELILOTI*

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Root nodule bacteria differ in their response to acidity. The inability of the prokaryotic symbiont to survive in acid conditions will be manifested as a lack of nodulation of the host leading to decreased legume productivity. *Sinorhizobium meliloti* is one of the most acid-sensitive root nodule bacteria, growing only down to pH 5.5, whereas *Rhizobium leguminosarum* bv *viciae* is far more acid-tolerant and can grow down to pH 5.0. An understanding of these differences is being investigated at the physiological and genetic level (Glenn *et al.,* 1999). One of the basic questions is whether acid-tolerant strains possess unique genes conferring acid-tolerance or whether they carry adapted versions of genes held in common with acid-sensitive strains.
Rhizobial mutants with acid-sensitive phenotypes have been identified using insertional inactivation of genes in *S. meliloti* and *R. leguminosarum.* The acid-sensitive mutants *R. leguminosarum* bv. *viciae* WR1-14 and *Sinorhizobium meliloti* RT3-27 were constructed by Tn5 mutagenesis of the wild-type strains WSM710 and WSM419, respectively. These two mutants belong to a "calcium-irrepairable" subclass of acid-sensitive mutants whose acid tolerance cannot be restored by high concentrations of calcium. Each mutant contains a single copy of Tn*5* inserted within a gene (*actP*) encoding a P-type ATPase that belongs to the CPx heavy metal transporting subfamily. Both WR1-14 and RT3-27 show sensitivity specifically to the heavy metal copper; omission of copper from low pH buffered media restores acid tolerance to these strains. The acid- or copper -sensitive defect in both *R. leguminosarum* and *S. meliloti* can be complemented by a plasmid clone containing the *actP* gene from *S. meliloti.* A *R. leguminosarum actP-gusA* fusion was transcriptionally regulated in a copper-dependent manner in *R. leguminosarum* but not in *S. meliloti.* The fusion is activated specifically in response to copper and not to any other heavy metal. In both organisms, downstream to the site of Tn*5* insertion is a gene (*hmrR*) encoding a putative heavy metal regulator. The precise function of the HmrR protein in either *Sinorhizobium* or *Rhizobium* has not been determined. Two more partial open reading frames were identified in the *R. leguminosarum* DNA sequence, one upstream to *actP* encoded an enzyme (TreY) possibly required for trehalose biosynthesis and another downstream to *hmrR* encoded an extracytoplasmic sigma factor (EcfR). Additional studies using EcfR mutants *R. leguminosarum* and *Sinorhizobium* should provide an indication of the importance of this protein in cell response to stress. Furthermore, the role of EcfR in the regulation of low pH-inducible genes such as *phrR* (Reeve *et al.,* 1998) and *lpiA* (Reeve *et al.,* 1999) can now be examined. The significance of *actP* and its adjacent genes in the stress response of root nodule bacteria will be presented.

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THE EXPRESSION OF URICASE GENES (NOD-35) IN UREIDE- AND AMIDE-TYPE LEGUME PLANTS AND THEIR MOLECULAR EVOLUTION

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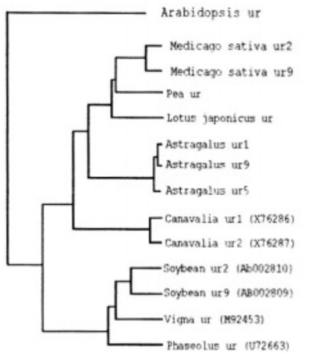
Biochemical and genetic research approaches have been paid to answer the question why ureide was overproduced in nitrogen fixing nodules of certain legume plants like *Vigna, Glycine* and *Phaseolus* sp. To compare the gene expression of enzymes contributing for ureide formation between ureide- and amide-type legume plants, we are going to clone uricase genes from both type of legumes and compare the gene expression and promoter activity.

In *Medicago sativa,* two uricase cDNA clones (ur2 and ur9) coding 308 ammo acids were isolated from a cDNA library and both genes were expressing in almost all tissues in low but similar magnitudes. In *Lotus japonicus,* one uricase cDNA clone coding 306 amino acids was obtained and was expressing in various tissues like *Medicago sativa* but the magnitude of the expression was highest in nodules and vascular bundles. Interestingly *in situ* hybridization analysis showed these uricase genes were expressing mainly in uninfected cells in nodules as in soybean nodules.


Arabidopsis ur
Medicago sativa ur2
Medicago sativa ur9
Pea ur
Lotus japonicus ur
Astragalus ur1
Astragalus ur9
Astragalus ur5
Canavalia ur1 (X76286)
Canavalia ur2 (X76287)
Soybean ur2 (Ab002810)
Soybean ur9 (AB002809)
Vigna ur (M92453)
Phaseolus ur (U72663)

Phylogenetic tree for plant uricase polypeptides.

When amino acid sequences of presented and reported uricase genes were subjected to phylogenetic tree analysis, uricases from amide-type legumes and ones from ureide-type legumes were located in separated branches, suggesting their different metabolic molecular evolution.

ISOLATION OF ACID TOLERANT PEANUT (*ARACHIS HYPOGAEA* L.) RHIZOBIA FROM SOILS OF CENTRAL ARGENTINA.

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1. Introduction

Soil acidity may significantly reduce the symbiotic nitrogen fixation limiting Rhizobium survival and persistence in soils and reducing nodulation (Graham et al 1994). Recently it was determined that soils of Central Argentina (Córdoba) showed low content of organic matter and relatively low pH in all the analyzed horizons (Hammp et al 1997). Peanut is a very important crop in Argentina, since this country is the third in the world in peanut exportation. A detailed examination of composition of rhizobia peanut-nodulating native population in soils of Central Argentina has not been carried out yet. In this work, we present results on the isolation and characterization of native peanut-nodulating rhizobia from soils of Cordoba in Central Argentina.

2. Material and Methods

Peanut-rhizobia were obtained from plant nodules and soils from different field sites in Córdoba. YEMA was used for routine cultivation of rhizobia and determination of growth rate. Screening of the acid tolerance was performed in a minimal medium (Howieson 1985) buffered with MES and HEPES to adjust pH in 5.5 and 7m, respectively. nifD-PCR was done with primers O_1 y O_2 (Stolzfus et al 1997).

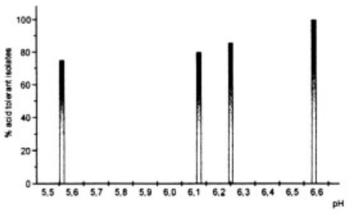
3. Results and Discussion

Table 1 shows the isolates, its origin and soil pH. Approximately 63% of the soil samples had pH below 6.5. A 70% of the isolates showed the 390 pb band of nifD-PCR and 93.5% of them were fast grower. In figure 1 we observe that there were no relationship between soil pH ant the acid tolerance of the rhizobia obtained.

Table 1

Isolates	Soil Location	Soil pH
NMA10	Malena	5.4
NLH24, NLH22, NLH22´, NLH27, NLH23, NLH23´, NLH25	Las Higueras	5.68
NCHA35A, NCHA32A, NCHA33B, NCHA30, NCHA31, NCHA22	Charras	6.25
Nale	Alejandro	6.38
NVAM22, NVAM24, NVAM32A, NVAM21	Villa María	6.38
NET30, NET31, NET36, NET33, NET37, NET39, NET32	Etruria	6.50
NBE24, NBE27	Bengolea	6.73
NOD31	Río Cuarto	6.80
NTI31	Ticino	8.19

Figure 1



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OSMOTIC STRESS EFFECTS ON PEANUT MICROSYMBIONTS

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1. Introduction:

It is well known that rhizobial growth is impaired by unfavorable environmental conditions, such as osmotic stress. One common mechanism of adaptation to osmotic stress is the accumulation of compatible organic solutes. We reported (Ghittoni, Bueno, 1996; Dardanelli, 1997) trehalose accumulation in some peanut rhizobia grown under hyperosmolarity imposed by NaCl. The objectives of this research were to compare those effects with the responses of bacterial growth to a nonpermeating solute (PEG).

2. Material and Methods

Bacterial strains recommended as peanut inoculants were kindly provided by international collections: TAL 1000 (NifTAL-USA) and USDA 3187 (IPAGRO- Brazil). Bacterial were routinely grown in Vincent YEM basal medium (1970). Osmotic strength of the medium was increased by adding either 0.05 or 0.40 M NaCl or 160g PEG-8000. Bacterial growth was turbidimetrically measured. Determinations: biomass by dry cellular weight after lyophylization, protein content of cells by the method of Bradford (1976), intracellular K^+ as described by Bernard et al (1993) and trehalose according to Müller et al (1992). Low molecular weight saccharides were extracted and quantified, and trehalose was identified as described by Dardanelli et al (1997).

3. Results and Discussion

USDA 3187: When grown in the presence of either PEG 8000 or NaCl, bacterial growth was negatively affected, biomass production and protein content of cells were also decreased, while cellular trehalose was significantly increased. Under hypersalinity, the rise in the trehalose content of cells was very striking. The disaccharide behaved as a compatible solute.
TAL 1000: In the presence of PEG 8000 in the medium, bacterial growth was diminished, biomass production was decreased and K+ cellular content was highly increased. Changes were also observed in the periplasmic low molecular weight saccharides and in the EPS content, accompanied by the disappearance of cellular trehalose. Biomass production was diminished and trehalose cellular content was augmented when grown under hypersalinity. Results suggest different mechanisms of osmoadaptation.

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A MODIFIED COLONIZATION PATTERN OF CEREALS BY *Azospirillum brasilense* Cd UNDER SALINE STRESS

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1. Introduction

Two objectives were followed in the present study: a). To analyze the effect of saline stress (300 mM NaCl) on the colonization efficiency of cereals by Azospirillum brasilense Cd. Para-nodules on roots were induced with 2,4-D to establish if these structures could act as bacterium protected niches, b). To study the involvement of the lipopolysaccharides (LPS) in the Azospirillum brasilense Cd-plant association by obtaining a Tn5-induced mutant. \\

2. Material and Methods

a). Seeds of *Triticum aestivum* cv. Buck charrua-INTA were germinated as previously described (Jofré et al., 1998). Individual seedlings were inoculated with control and stressed *Azospirillum brasilense* Cd::(*nifA- lacZ*) (2.2-2.4 x 10^6 cfu per milliliter of hydroponic solution). 0.4 µg/ml 2,4-D was added. Colonization studies were done two weeks after inoculation; roots were taken from seedlings and stained as described by Arsène et al. (1994).

b). A Tn5 mutant lacking the O-antigen in the LPS was obtained (Vanstockem *et al.,* 1987). LPS were analyzed by SDS-PAGE (Laemmli, 1970; Tsai and Frash, 1982). Colonization of *Zea mays* was determined as above, with wild and mutant strains (2 x 10^7 ufc/seedling), in hydroponic medium added with 200 mM NaCl.

c). DNA isolation, digestion, ligation, electrotransformation, cloning and PCR were done by standard methods.

3. Results and Discussion

a) Control bacteria were distributed along the whole root system of *Triticum aestivum,* except the elongation zone. Stressed bacteria localized mainly at the root tops and the lateral roots, Both, control and stressed bacteria colonized the *para*-nodules. These results could be related to a modification of the *Azospirillum* polysaccharide composition.

b) The LPS rough mutant obtained did not colonize maize roots as efficiently as the wild type under standard conditions. Nevertheless, under saline stress, the mutant was more efficient than the wild-type Cd. Reverse mutagenesis produced the same phenotype than the Tn5 mutant.

This is the first report involving the O-antigen of LPS in the colonization of cereals by *Azospirillum brasilense* Cd.

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BALANCING THE C/N RATIO OF TERRESTRIAL ECOSYSTEMS IN A CO_2-RICH WORLD: EXAMPLE OF AN ECOSYSTEM CONTROL OF SYMBIOTIC N_2 FIXATION

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Trifolium repens, grown in pots in silica sand and supplied sufficiently with a rich nutrient solution, showed a 40% increase in biomass production when grown under elevated atmospheric pCO_2 as compared to ambient conditions. The growth-induced increase in N demand was covered by a parallel increase in symbiotic N_2 fixation and increased assimilation from mineral N, i.e. the percentage of N derived from symbiosis did not change (Zanetti et al. 1998). When the clover grew under field conditions under elevated atmospheric pCO_2 (Swiss FACE), biomass production increased similarly. However, an evaluation of the N-sources (^{15}N isotope dilution technique) clearly showed that under elevated atmospheric pCO_2 all nitrogen that was additionally assimilated in clover came from symbiotic N_2 fixation and none from mineral N, i.e. the percentage of N derived from symbiosis increased (Zanetti et al. (1996). Under the same field conditions, grasses showed increased symptoms of N limited growth under elevated atmospheric pCO_2 (Fischer et al. 1997; Zanetti et al. 1997; Rogers et al. 1998). These data suggest that the 60% increased leave photosynthesis (CO_2 fixation) under elevated atmospheric pCO_2 (Rogers et al. 1998) caused increased N-sinks in the whole ecosystem. To fill these N sinks, not enough mineral N may have been available to meet the increased N demand of the more intensively growing plants under elevated atmospheric pCO_2. This made the legumes to depend more on symbiotic N_2 fixation and at the same time made them more competitive against grasses under elevated atmospheric pCO_2 (Zanetti et al. 1996; Hebeisen et al. 1997). Strikingly, symbiotic N_2 fixation increased under elevated atmospheric pCO_2 also by 60% in the clover/grass mixed community, thus apparently maintaining the C/N ratio of plant material at the sward level (Zanetti et al. 1996; Hartwig et al. 1999). Beside plant biomass, new or increased N sinks may also be located in increased rates of denitrification (Ineson et al. 1998), increased C sequestration into soil organic matter (Nitschelm et al. 1997; Van Kessel et al. 1999) or changes in soil biological activity as indicated from changes in the soil microbial community structure (Marilley et al. 1999; Montealegre and Sadowsky, unpublished data). It is concluded that increased C input into the ecosystem under elevated atmospheric pCO_2 is counterbalanced by appropriately increased symbiotic N_2 fixation, thus maintaining the C:N ratio at the whole ecosystem level. This response clearly demonstrate that symbiotic N_2 fixation may be co-driven by whole ecosystem interactions.

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EFFECTS OF LEGUMES CULTIVATION ON SYMBIOTIC POTENTIAL AND COMPETITIVENESS OF BRADYRHIZOBIUM JAPONICUM

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1. Introduction

It is well known that biological nitrogen fixation(BNF) by rhizobia plays an important role in production of high protein food sources and nitrogen cycle in soil ecosystems. The relative contribution of symbiotic N_2 fixation to the global total is about 70% (Paul, 1988). Because of these benefits of symbiotic relationship between legume and *Rhizobium,* mutipurpose legumes such as grain, forage, and green manure have been used for cropping systems resulting in the maintainance of soil fertility and productivity. In legume-*Rhizobium* symbiosis, the symbiotic potential of rhizobia indicates soil productivity by them (Brockwell et al., 1988 ; Kang, 1998) and suggests the beneficial effect of BNF to alleviate the various problems which originate from chemical fertilizer consumption to meet nitrogen demand of host plant as a protein source (Bohlool et al., 1992). The symbiotic potential in soils is generally determined by the population size, N_2 fixing ability, and host specificity of soil rhizobia. It is frequently said that soil microbial community is affected by root exudates of a cropping plant (Hiltner, 1904; Vancura, Hovadik, 1965). In this respect, this experiment was carried out to elucidate effects of the legumes cultivation on symbiotic potential and competitiveness of indigenous *Bradyrhizobium japonicum* to upland soils.

2. Materials and Methods

For the experiment, leguminous crops of soybean (*Glycine max.* cv. Danweonkong), peanut(*Arachis hypogaea* cv. Daegwangtangkong), and alfalfa (*Medicago sativa* cv. Vernal) were cultivated for three years in a Alluvial clay loam soil of pH 5.1 and 1.6×10^4 cfu *Bradyrhizobium japonicum* per g of soil. The symbiotic potential and competitiveness of soil populations of *B. japonicum* were evaluated by inoculating soybean with 10-fold-diluted soil suspensions (Kang et al., 1991). Soybean was grown in modified Leonard bottle-jar, in which horticultural-grade vermiculite was contained as a rooting medium and N-free nutrient solution was supplied. In the competition studies, *gus*-marked *B. japonicum* strain YCK 141 and USDA 110 as competing pairs of soil inocula were introduced at the same cell levels with those of soil populations. Plants were thinned to two per jar at day 7 after planting. At harvest (day 35), nodules and shoot dry weight were measured. Nodule occupancy by soil populations was determined by GUS assay of the competing strain nodules (Kang et al., 1997). On the one hand, effects of legumes cultivation on the growth of *B. japonicum* was investigated in nutrient solution as well as in field soils.

Results and Discussion

Populations of *B. japonicum* were increased by cropping and were highest in soils which had been cultivated with peanut followed by soybean and alfalfa showing 108.7×10^3, 74.0×10^3, and 68.3×10^3cells/g·soil, respectively. Some serogroup strains of *B. japonicum* also grew well in the nutrient solution which had been cultivated with peanut. The symbiotic potential of soils with *B. japonicum* in terms of shoot production of soybean was highest in soybean soils and was not different between peanut and alfalfa soils. However, nodule number and nodule mass were in order of alfalfa soils > soybean soils > peanut soils. Nodule occupancy of soil populations

EFFECTS OF ENVIRONMENTAL STRESSES ON THE EXPRESSION OF NODULATION GENES OF BEAN RHIZOBIA STRAINS ISOLATED FROM SOILS OF ARGENTINA, BRAZIL AND SPAIN

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Common bean (*Phaseolus vulgaris*) is the most important legume in human nutrition, being a source of many proteins and essential amino acids. Bean crop is very important in Argentina, Brazil and Spain and an increase in the grain productivity would certainly have impacts on the human nutrition and on the regional economies.

Symbiotic interaction between *Rhizobium* strains and leguminous plants leads to the formation of nitrogen-fixing root nodules. This interaction starts with an exchange of chemical signals between the symbiotic patterns. The plant signals, flavonoids exuded by roots, activate the expression of nodulation genes, resulting in the production of the rhizobial lipochitooligosaccharide signals (Nod factors). This signal interchange leads to nodule formation processes and subsequently to nitrogen fixation. There is a great variety of rhizobia nodulating *Phaseolus vulgaris,* like *R. leguminosarum* bv. phaseoli, *R. tropici, R. etli, R. giardinii* and *R. gallicum.*

Symbiosis establishment and nitrogen-fixing nodule formation are influenced by many environmental factors, such as soil pH and temperature. In this work we have used a broad collection of rhizobia isolated from bean producing areas in Argentina, Brazil and Spain. The ability to grow *in vitro* at high temperature (above 35°C) and in buffered media (acidic and alkaline) was determinated. Bean nodulating rhizobia showed a great variety of response to stressful conditions. All *R. tropici* strains could grow at 38°C. *R. tropici* strains were also the only ones able to grow at pH 5. Nod factor production was tested both in optimal and stressful conditions. TLC analyses revealed that *Rhizobium* strains nodulating *Phaseolus vulgaris* produce different Nod factors profiles. PCR analysis allowed us to establish a correlation between the presence of fast-migrating bands and the presence of sulphation *nod* genes (*nodH*). Environmental stressful conditions affected both the amount and profile of Nod factors.

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INOCULATION OF COWPEA PLANTS GROWN UNDER IRRIGATED CONDITIONS WITH RHIZOBIA STRAINS ISOLATED FROM SOILS OF THE SEMI-ARID REGION OF BRAZIL.

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Cowpea is an important staple food cultivated throughout the semi-arid region of the Brazil. The crop is generally grown by peasant farmers without irrigation. More recently significant areas with irrigation facilities have been planted with cowpeas. Under such conditions the soil rhizobium population is stimulated and may impair the establishment of selected efficient strains introduced by seed inoculation. Selected strains isolated from different soils of the semi-arid region of Pernambuco State, were evaluated for efficiency and tested as inoculant to cowpeas planted in the Irrigation Settlement of Bebedouro, Petrolina, Pe. The soil of the experimental area already contained a significant native rhizobium population. Seed inoculation with some strains significantly improved plant nodulation but plant dry matter production was not directly related to nodulation. One of the successful strains has the ability to produce bacteriocins under plate conditions. Bacteriocin production has been related to improved competitive ability. The use of competitive and efficient strains of rhizobium may provide an adequate supply of fixed nitrogen to improve plant growth and grain yields.

Financial support: PRODETAB

SECOND YEAR RHIZOBIA INOCULATION OF COWPEA ON A NON-IRRIGATED FIELD AT THE SEMI-ARID BRAZILIAN REGION

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The capacity of diazotrophic bacteria to survive in soil and to compete with the native population are the main factors determining the quality of an efficient inoculant. The behavior of Rhizobium strains isolated from nodules of cowpea (Vigna unguiculata L.Walp.) cultivated in soil samples from the Brazilian semi-arid region was evaluated in the second year of an experiment on a non-irrigated area of the Experimental Station at Embrapa Semi-árido (Petrolina, PE). The objective of this study was to select Rhizobium strains to be used as inoculant for cowpea cultivated in non-irrigated area of the semi-arid region of Brazil. Rhizobium strains were evaluated for BNF efficiency when used as inoculant for cowpea cultivated in a non-irrigated area of the experimental station at Embrapa Semi-árido, Petrolina – Pe, Brazil. The experiment was performed as a randomized complete block design with 2 factors and 3 replicates. Factor 1 consisted of the frequency of inoculation used: (1) inoculation during the **1st** year, (2) inoculation during the **2nd** year and (3) inoculation during the two consecutive years. Factor 2 consisted of 11 selected Rhizobium strains isolated from different soils of the North-east region of Brazil. Plants were harvested at 30 days after germination (DAG), during flowering (45 DAG) and at 65 (DAG).

A preliminary experiment was performed in a greenhouse to evaluate the native rhizobia population. Cowpea plants grown on soil samples collected during the second year of experiment, showed an increase on nodule number, suggesting an increase in the native population capable of nodulating cowpea. In both treatments, inoculation during the second year and inoculation during the two consecutive years showed an increase in nodule number when compared to inoculation during only the first year. The results indicate a partial infective capacity of these strains which will be confirmed later by immunological tests. These data indicate the importance of inoculation in marginal areas where, during the dry period, Rhizobium population is drastically diminished favouring nodulation by the inoculant strain.

Acknowledgement

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ALUMINIUM TOXICITY ON PEANUT SYMBIONT IN THE ACIDIC ENVIRONMENT

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1. Introduction

Aluminium (Al) constitutes abouts 8% of the earth crust. In acid soils, Al toxicity and acidity probably limit rhizobial growth more than manganese toxicity and calcium deficiency (Keyser, Munns, 1979). The combination of acidity and Al harms the development of root nodule bacteria which then unfavourably influences both soil and host-plant root colonization. Biotransformation is one defense strategy used by microbes subjected to high levels of metallic elements in their ecological niches (Silver, 1989). The aims of this study were to evaluate the effects of high concentrations of $AlCl_3$ and low pH on the growth of *Bradyrhizobium sp.* (peanut symbiont) and to investigate whether the molecule glutathione (GSH) and glutathione S-transferase (GST) activity are involved in the biotransformation mechanism under this environmental stress.

2. Results and Discussion

The strain was Bradyrhizobium SEMIA 6144 (peanut recommended inoculant) and the growth medium was MSM (Brown, Dilworth, 1975) at different pH (7.0, 6.0, and 5.0). At the lowest pH, different $AlCl_3$ concentrations were used (12.5, 25, 50, 100 μM). The GSH content (Tziete, 1969) and the GST activity (Vessey, Boyer, 1984) were determined. The results of the bacterial growth rate demonstrated a significant decrease (60 %) at pH 5.0 with respect to pH 7.0 at 48 h of culture. A greater decrease at pH 5.0 and 50 μM Al^{3+} was observed compared to acid pH per se (Fig. 1). The GSH content was significantly increased 3-fold (Fig 2) and the GST activity was also increased 9-fold under the same conditions.

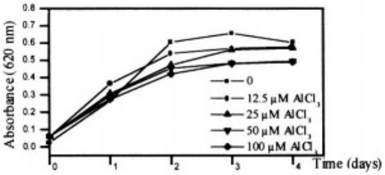

Fig 1. *Bradyrhizobium* sp. growth rate at pH 5.0 and different concentrations of $AlCl_3$.

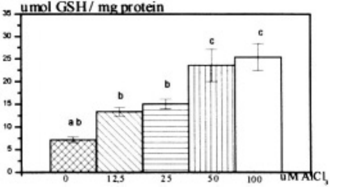

Fig 2. Glutathione content in *Bradyrhizobium* sp. at pH 5.0 and different concentrations of $AlCl_3$.

In conclusion, *Bradyrhizobium* sp. is a strain sensitive to aluminium and the increment of the GSH content and the GST activity would be related to the defense mechanism of the bacteria under stress condition induced by high concentrations of Al^{3+} and acid pH.

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CHARACTERIZATION OF SALINITY-TOLERANT *BRADYRHIZOBIUM SP* STRAINS

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Environmental stress plays an important role in crop yields of legumes grown in Latin America. Salinity is considered a governing stress factor in the normal distribution of plants and an increasing problem as the surface of unfavorable farming areas expands.

Salinity-tolerant *Bradyrhizobium* sp strains (88 mM, 264 mM y 440 mM NaCl) were characterized according to: Use of 95 sources carbonated by the BIOLOG GN method, type of metabolism, determination of exopolysaccharides, indol acetic acid production and/or analogues, siderophores production, phosphate solubilization, antibiotic resistance, plasmid profile and protein and lipid profiles. In general, the salinity-tolerant strains increased their adaptive capacity when compared to the native strains by showing larger C-sources-oxidation capacity, increase in growth rate and production of exopolysaccharides involved in adherence. Some tolerant strains lost a high-molecular-weight plasmid, suggesting that salinity may behave as curing agent. Alterations at membrane level were observed when total lipids, proteins and carbohydrates were determined.

Tolerant strains are efficacious in nodulating up to 254 mm-NaCl-salinity range and some of them have acquired PGPR capacity lying therefore promising strains for unfavorable areas.

SECOND YEAR RHIZOBIA INOCULATION OF COWPEA ON AN IRRIGATED FIELD AT THE SEMI-ARID BRAZILIAN REGION

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The use of biotechnology is opening everyday new perspectives such as inoculation practices in agriculture with rhizobia or other microorganisms. The behavior of rhizobia strains isolated from nodules of cowpea (*Vigna unguiculata* L.Walp.) cultivated in soil samples from the brazilian semi-arid region was evaluated on a second year of an inoculation experiment on an irrigated area of the Experimental Station of Embrapa Semi-árido (Juazeiro, BA). The inoculation with the strain 8-4 increased the grain yield from 1250 $kg.ha^{-1}$ to 2100 $kg.ha^{-1}$ (control), suggesting that despite this plant to be recognized as not having specificity in relation to the rhizobia, it is possible to select a rhizobia capable of competing with native population, therefore increasing production at the expenses of BNF only.

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XYLEM SAP AMINO ACID COMPOSITION OF SOYBEAN AFTER TRANSFER TO WATER-CULTURE.

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The transfer of nodulated soybean plants from vermiculite to aerated water-culture for 4 days resulted in strongly diminished ureide transport in the xylem sap. Despite little change in the total amino acid content of the sap, marked changes occurred in the composition. A large increase in aspartic acid (Asp) and decrease in both asparagine (Asn) and glutamine (Gln) occurred. Supplying nitrate in the water-culture medium reduced the fall in Asn and increase in Asp. Non-nodulated plants grown in vermiculite with nitrate also showed increased Asp and decreased Asn in the xylem sap on transfer to N-deficient water-culture but not when nitrate was maintained during water-culture. The increased Asp/Asn ratios in the xylem sap suggest an alteration in Asn metabolism and appear to be associated with treatments that diminish N assimilation (whether nitrogen fixation or nitrate assimilation). Prominent levels of Gln were only seen in the xylem sap of plants with active nodules (i.e. high xylem ureide levels). Despite its relatively low level in nodule tissue, evidence suggests that much of the xylem sap Gln may have originated in the nodule.

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ACID-TOLERANT AND FAST-GROWING SYMBIOTIC BACTERIA ISOLATED FROM SOYBEAN NODULE IN TROPICAL ASIA

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A strain of root nodule bacteria (strain BjGJ3a), isolated from cultivated soybean nodules in central Java, exhibited a very fast growth rate and high acid tolerancy. The generation time of this strain was only 34 min at pH 7.0 compared to 400min for several standard strains of *Bradyrhizobium japonicum.* When the nutrient medium was prepared at pH 3.5 with HC1 (unbuffered medium), the growth of strain BjGJ3a started 40 hrs after inoculation, whereas the cell growth started within 4 hrs at pH 7.0. The pH value of culture medium increased to pH 7.5 during cell growth. The bacterial growth was also confirmed when the medium was prepared at buffered condition (pH 5.0 with 0.1 M of MES buffer addition). During cell growth in acidic condition, this strain may exude some substances which affects a pH increase in the culture supernatant. This behavior may function to neutralize the cell environment to keep the neutral condition to perform infection of the host plant.

Nineteen kinds of enzyme activity were examined semi-quantitatively by APIZYM test plate. The strain BjGJ3a exhibited a unique profile of enzyme-activity patterns compared to three other strains. Particularly, activities of **□-galactosidase**, **□-glucosidase** and N-acetyl glucosaminidase were high. The optimum pH of these enzymes are in the acidic range. All tested strains expressed acid phosphatase when the bacteria were cultured at pH 7. Except for strain BjGJ3a, this enzyme activity disappeared on culturing at pH 5. Strain BjGJ3a kept acid phosphatase activity even at pH 3.5.

Partial sequences of 16S rRNA gene were analyzed to identify the genus and species of strain BjGJ3a. After PCR, the amplified fragment was cloned and applied for sequencing analysis. The data were analyzed by a homology search on the data base of DDBJ, and the strain BjGJ3a was identified as Enterobacteriaceae but not Rhizobiaceae. Further analysis, such as fatty acids content, and reference to the data base of MIDI, also indicated that strain BjGJ3a was Enterobacteriaceae with more than 90% similarity. Another sequence of a PCR fragment (300 bp) amplified with the primers for *nodC* gene (Ueda et al, 1995) was analyzed. From homology search of the data base, the strain BjGJ3a contains a part of *nodC* gene fragment at 91% similarity with *Bradyrhizobium elkanii* and 80% similarity with *B. japonicum.* This fact suggests that nodulation genes might transfer from *Bradyrhizobium* strains to the Enterobacteriaceae (strain BjGJ3a) in the rhizosphere. The loci of the *nod* genes (plasmid or chromosomal location) and the sequence of other *nod* genes of BjGJ3a are under examination. The strain BjGJ3a exhibited symbiotic character on soybean host (*Glycine max* cv. Peking) though it is from the Enterobacteriaceae. How could the symbiotic genes transfer from Rhizobiaceae into Enterobacteriaceae, and how can strain BjGJ3a express symbiotic genes? Horizontal transfer of symbiotic genes (symbiotic island) could have easily happened in the rhizosphere even among bacteria belonging different genera.

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INFLUENCE OF IRON STARVATION IN NODULATION COMPETITIVENESS OF RHIZOBIA.

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Competition among microbes present in habitats like soil and rhizosphere can be influenced by the availability of nutrients. Although iron is abundant in nature, the extremely low solubility (10^{-18} M of Fe^{3+} at pH 7), force bacteria to develop different strategies to obtain enough iron from their environment. Most bacteria - including rhizobia- are able to produce special compounds termed siderophore to acquire this essential nutrient. Certainly, the most studied example of the ecological significance of siderophore-mediated competition resides in rhizospheric- pseudomonads.

In this work we evaluate the effect of iron-starved inoculum and siderophores utilisation in the infectiveness of rhizobia. Competition experiments were designed using a native strain of *Sinorhizobium meliloti* and two isogenic mutants defective on siderophore mediated iron acquisition systems. Different mixtures of iron-limited and iron-sufficient cultures were used to inoculate alfalfa plants.

Results obtained from this work show that: a) when plants were co-inoculated with cells grown on iron-starved medium, the wild type strain exhibited greater nodule occupancy than the mutant strain; b) an increase on iron availability in the inoculant medium, led to an increase in nodule occupancy.

When the cells were grown on the iron-starved medium used to inoculate the plants, the impaired growth could be overcame by $FeCl_3$ addition to the media. Besides, at the EDDHA concentrations used in this medium, the rhizobial siderophore is excreted. It is well known that siderophore production is regulated by the intracellular iron content. Taking into account these observation, we can conclude that inoculum prepared from iron-limited medium are iron starved. Therefore, results obtained lead to the following main conclusion: the iron status of the cells used in the inoculant mixture affect the nodulation competitiveness of rhizobia.

High affinity iron acquisition systems in Sm 242 not only involve its own siderophore, different compounds- ferrichrome, hemin, leghemoglobin- can be used as iron sources as well. Those microorganisms which possess highly efficient systems for iron acquisition will be able to acquire this metal more effectively. This characteristic would confer competitive advantage to these strains in soil and in the rhizosphere conditions where iron bioavailability is naturally limited.

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REGULATION OF CATALASES DURING FREE-LIVING GROWTH OF *SINORHIZOBIUM MELILOTI* AND THEIR PROTECTIVE ROLE DURING SYMBIOSIS

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Both partners of *Medicago-Sinorhizobium* symbiosis produce reactive oxygen species (ROS) in normal functional nodule like all aerobic organisms. Catalases are key enzymes involved in the detoxification of ROS by the dismutation of H_2O_2 in O_2 and H_2O. Analysis of catalase activity on native gels showed that *S. meliloti* produces at least three catalases, namely two monofunctional ones (KatA and KatC) and a bifunctional catalase/peroxidase (KatB). A differential regulation of catalases was observed during growth of free-living bacteria on M9 minimal medium. The highest levels of KatA activity were found during the exponential phase, with a gradual decrease during stationary phase. Since KatA is the unique catalase which is inducible by H_2O_2, these results suggest that *S. meliloti* has to deal with a burst of H_2O_2 production during late exponential growth. KatC activity was only detected during the stationary phase and increased as KatA activity decreased. Only KatB activity was constant. The *katA* gene was previously cloned (Hérouart et al, 1996) and used as probe to isolate the *katC* gene (Sigaud et al., 1999). Analysis with *katC::lacZ* indicated that growth-dependent KatC regulation is mainly or solely transcriptional. Moreover, an induction of *katC* was also observed after bacteria had been exposed to heat stress (37°C), salt stress (4 % NaCl), paraquat (10^{-3}M) or ethanol for 1h, indicating that the *katC* expression profile is very similar to the one of many genes encoding monofunctional HPII catalases.

To investigate the role of *KatA* and *KatC* during the development of nitrogen fixing nodules, two simple *katA* and *KatC* mutants and a *katA katC* double-mutant were constructed. *M. sativa* host plants were inoculated with these three mutant strains and with the wild-type Rm5000 strain as a control. Similar efficiencies of nodulation and nitrogen fixation capacities were observed for the plants inoculated with the single mutants and with Rm5000. However, only 75 % of the plants inoculated with the double-mutant showed a nodulating phenotype and a dramatic decrease of nitrogen fixation was observed.

The localisation of H_2O_2 was performed in nodules of plants inoculated with the wild-type Rm5000 strain using $CeCl_3$ which precipitated in presence of H_2O_2. Examination by transmission electron microscopy of 6-week-old nodules showed that H_2O_2 was not detected in bacteroids, indicating the high efficiency of H_2O_2-scavenging systems. In contrast, a strong accumulation of H_2O_2 was observed in senescent infected plant cells, especially close to the peribacteroid membranes.

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SECTION XI

BIOLOGICAL NITROGEN FIXATION IN SUSTAINABLE AGRICULTURE

BIOLOGICAL NITROGEN FIXATION: PHOSPHORUS - A CRITICAL FUTURE NEED?

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1. Introduction

The importance of biological nitrogen fixation (BNF) to world food security is unquestionable. Since the dawn of farming, symbioses capable of BNF have been instrumental in both supplying food and improving soil health (van Kessel, Hartley 2000). Today Earth's 6 billion people consume an average of nearly 11 g N per day or about 24 Mt annually (Fink et al. 1999). Worldwide legumes provide at least 33% of humankind's N needs, however, in the tropics and subtropics plant sources provide much more (up to 80%) of the dietary N requirements. Legumes are grown on approximately 275 million hectares or nearly 11% of the arable land (van Kessel, Hartley 2000; Kinzig, Socolow 1994).

The use of crops capable of symbiotic nitrogen fixation is a primary component in agricultural sustainability. Nitrogen acquired from BNF is essentially "free" N to be used by the host plant, by an associated crop, or subsequently by a crop in rotation. Although the benefits of BNF to the improvement of impoverished soil in developing countries is obvious, its importance to intensive agriculture in developed countries has diminished due to easy access to inexpensive N fertilizer (Graham, Vance 2000). However, even modest use of alfalfa in rotation with corn in the U.S. could substantially reduce N fertilizer need (Peterson, Russelle 1991). Moreover, biologically fixed N incorporated directly into organic matter is much less susceptible than fertilizer N to volatilization, denitrification, and leaching. Thus, potential contamination due to loss of N to the environment would be reduced.

With the Earth's population increasing at 1.4% annually and expected to reach 10 billion by 2040 (Mannion 1998; Vance 1998), unprecedented increases in crop production will be needed to maintain current levels of dietary protein and caloric intake. This enhanced production must be achieved despite continued deterioration and loss of prime agricultural land, essentially static yields in many areas of crop production, and exacerbation of the primary constraints limiting crop production. The enhanced use of crops capable of BNF offers promise to meet some of these challenges.

2. Phosphorus: A Constraint to BNF

Although BNF has and will continue to contribute significantly to world food security, achievement of maximum benefits is limited by availability of phosphorus (P) (Cocking, Kennedy 1997). Phosphorus is second only to N as the most limiting nutrient for plant growth (Holford 1997; Schactman et al. 1998). Crop production on 33% of the world's arable land is limited by P availability (Sanchez, Euhara 1983), with the acid weathered soils of the tropic and subtropics particularly prone to P deficiency. Smallholders in southern Africa frequently apply less P as fertilizer than is removed in the grain (Hedley et al. 1995; Snapp et al. 1999), and even where

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adequate P fertilization occurs, less than 15% of the applied P is taken up in the first year (Holford 1997). The concentration of free P in most soils (from 2-7 μM) is well below the Km for uptake (Bieleski 1973). An even greater reason for concern is that by some estimates, rock phosphate reserves could be depleted in as little as 60-90 years (CAST Report 1988; Runge-Metzger 1995).

Plants engaged in symbiotic nitrogen fixation generally have a high requirement for P (Robson 1983; Jungk 1998). This is thought to be related directly to the high ATP requirement for nitrogenase function (Ribet, Drevon 1995; Al-Niemi et al. 1997). However, several other processes involving P also contribute to this requirement including: enzyme activation-inactivation, signal transduction, carbon partitioning, plastid function, and membrane biosynthesis. Thus the P concentration in nodules is frequently significantly greater than that found in shoots and/or roots. For example, the P concentration of soybean and pea nodules on low to adequate P approximates 6 $mg \cdot g\ DW^{-1}$, while shoot P concentration is between 2-3 $mg \cdot g\ DW^{-1}$ (Jakobsen 1985; Israel 1987). Moreover, when N_2-fixing species are grown in low P conditions, P fertilization leads to increased nitrogenase activity, nodule number, nodule mass, and plant N accumulation (Andrew, Robins 1969; Gates 1974; Robson et al. 1981; Jakobsen 1985; Israel 1987). Whether the effect of P upon nitrogenase activity and nodule function is direct or is mediated by changes in shoot growth and metabolism remains uncertain. In clover (Robson et al. 1981) and pea (Jakobsen 1985) stimulation of N_2 fixation by P addition was mediated through rapid enhancement of shoot growth with a resultant influence on nodule parameters. Graham and Rosas (1979) found that P deficiency limited N_2 fixation by reducing shoot growth. By contrast, P deficiency in soybean and *Stylosanthes* appears to impact nodule function more directly than shoot function (Gates 1974; Israel 1993). More recent data from soybean suggest that nodule O_2 permeability is increased during P stress and wasteful respiratory pathways are activated (Ribet, Drevon 1995). Studies of the bean *Rhizobium tropici* symbiosis (Al-Niemi et al. 1997) have shown that bacteroids obtained from P-sufficient plants resembled P-stressed free living *Rhizobium* cells in having similar acid and alkaline phosphatase isoforms. The authors suggested that the inorganic P concentration in the symbiosome space can be low even in P-sufficient plants. Using a rapid protocol to isolate bacteroids and measuring *in planta* ^{32}P uptake, Al-Niemi et al. (1997) showed that *R. tropici* bacteroids from P-deficient plants had greater rates of P uptake than those in P-sufficient plants. Unfortunately, neither nitrogenase activity nor other nodule functions were measured in these experiments. Although *Rhizobium meliloti* requires a P uptake system for effective nodule formation (Bardin et al. 1996) and P stress adversely affects several aspects of rhizobial growth and metabolism (Smart et al. 1984; McDermott 2000), the quantity and nature of internal P supplied to nodule bacteroids needs further study.

3. Adaptations for Low P Environment

With increasing concerns about the long term supply of rock phosphate, the lack of P available to small landholders, and the importance of P to effective BNF, it seems prudent to consider strategies/adaptations that lead to improved P acquisition and use. To effectively adapt to a low P environment plants and bacteria have adopted two broad strategies: 1) those targeted to conserve use; and 2) those directed to enhance acquisition or uptake (Lajtha, Harrison 1995). Strategies that conserve use of P involve decreased growth rate, increased growth per unit P uptake, remobilization of internal P, modified carbon metabolism, and alternative respiratory pathways. By comparison, strategies that lead to better uptake include increased production of phosphatases, organic acids, P transporters and porin channels, and expanded surface area (Marschner et al. 1986;

Wanner 1990; Lajtha, Harrison 1995; McDermott 2000). Additionally, plants frequently develop symbioses with mycorrhizal fungi to enhance P uptake (Bolan 1991).

4. Proteoid Roots in White Lupin: Adaptations for Survival in a Low P Environment

Over the last few years we have become interested in white lupin as a model system to evaluate legume adaptations to P stress. White lupin can effectively acquire and use P, although it does not form a mycorrhizal symbiosis (Trinick 1977; Gardner et al. 1982). Instead, its adaptation to P stress is a highly coordinated modification of plant root development and biochemistry that results in the formation of proteoid roots with exudation of organic acids and acid phosphatases from proteoid root zones (Dinkelaker et al. 1989, 1995; Johnson et al. 1996a and b; Gilbert et al. 1999a and b; Watt, Evans 1999; Neumann et al. 1999). Proteoid roots are densely clustered tertiary roots (sometimes called bottle-brush roots) that arise from the pericycle opposite protoxylem points. Unlike typical lateral roots that arise at random along the axes of primary and secondary roots, proteoid roots develop laterals that emerge from every protoxylem pole within the axis. Proteoid roots are also densely covered with root hairs. Thus, root surface area can be increased more than 100-fold. Phosphorus uptake is greatly enhanced in proteoid root zones (Neumann et al. 1999). Proteoid roots develop in many species capable of BNF including *Acacia, Mirica, Alnus, Casuarina,* and *Hippophae* (Skene 1998).

As might be expected, auxins and auxin transport play a role in proteoid root development. Gilbert et al. (2000) have shown that auxin transport inhibitors block proteoid root formation in P-stressed white lupin, while auxin application mimics proteoid root formation in non P-stressed plants. Ethylene and IAA production were slightly increased in -P proteoid roots. An Arabidopsis mutant, *superroot,* that has increased levels of free and bound IAA displays excessive lateral root formation that resembles proteoid roots (Boerjan et al. 1995). Moreover, transgenic plants overexpressing IAA biosynthesis genes show a root phenotype similar to proteoid roots (Aeschbacher et al. 1994).

Phenomenal amounts of citrate and malate are exuded from P-stressed white lupin proteoid roots (Dinkelaker et al. 1989; Johnson et al. 1994). The resulting carbon can account for as much as 23% of the total photosynthetic carbon. Thirty to 40% of the carbon exuded from P-stressed proteoid roots is derived from enhanced expression of phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) (Johnson et al. 1994, 1996a, 1996b). The excretion of organic acids increases the availability of mineral-bound P by solubilizing Ca, Fe, and A1 phosphates (Dinkelaker et al. 1995) and releasing soluble organic P bound to humic substances by Fe and Al (Braum, Helmke 1985). Organic acid exudation under P stress is not limited to lupin, in fact, numerous legume and other species capable of BNF release large quantities of organic acids (Jones 1998; McLay et al. 1997). Organic acid exudation is also a response to the presence of toxic levels of Al. Another adaptation for obtaining scarce P that occurs in proteoid roots is the synthesis and exudation of novel acid phosphatases. Both extracellular and intracellular acid phosphatases are ubiquitous in plants and increases in acid phosphatase activity under low P conditions have been demonstrated for many plants (Goldstein 1992; Duff et al. 1994) Acid phosphatases exuded into the rhizosphere can hydrolyze monoester soil organic P. Such organic P esters can account for 30-80% of the total P in agricultural soils (Gilbert et al. 1999b). Tadano and Sakai (1991) showed P-stressed lupin secreted 20-fold more acid phosphatase from roots than nonstressed controls. Recent studies from our laboratory (Gilbert et al. 1999a) have shown that white lupin synthesizes a novel acid phosphatase in proteoid root zones of -P-stressed plants, and this novel enzyme is secreted into the rhizosphere. Synthesis of the new acid phosphatase was dependent upon low internal P concentrations. Acid phosphatases and phytases are exuded from P-stressed roots of many legumes including: soybean, common bean, clovers, alfalfa, and *Stylosanthes* (Duff et al. 1994; Li et al. 1997).

While most reports document that low P conditions inhibit nodulation and nitrogen fixation, preliminary experiments with low P-stress white lupin indicate that nodulation is stimulated, particularly in proteoid root zones (G.A. Gilbert, S.J. Temple, D.L. Allan, C.P. Vance unpublished). Total nodule number is increased by 54% in P-stressed white lupin with 80% of these nodules located in proteoid root zones. The prolific development of tertiary lateral roots in proteoid zones appears to increase the plant's susceptibility to nodulation. Whether this increased nodulation is related to enhanced exudation of organic compounds or to the emergence of numerous tertiary lateral roots remains to be investigated. We need to determine the amount and the type of flavonoids and isoflavonoids released in proteoid root zones and how this influences nod gene expression in rhizobia.

To further understand the biochemical and developmental adaptations that lead to and occur in proteoid roots we have initiated a molecular approach. To date we have isolated approximately 200 expressed sequence tags (ESTs), with a goal to obtain at least 1,000. Several proteoid root ESTs encode enzymes involved in citrate and malate synthesis including: citrate synthase, MDH, and PEPC. Several other ESTs appear to be involved in signal transduction, ion or water transport, and phosphorylation reactions. We anticipate that this approach will facilitate the identification of plant genes that may be used in biotechnological approaches to improve P acquisition.

5. Enhancing Phosphorus Availability in Cropping Systems

A substantial body of evidence indicates that growing legumes as intercrops and in rotations can increase P availability. For example, intercropping wheat with lupin in low P soils resulted in a doubling of wheat dry matter because of improved P nutrition. The P uptake rate was at least 1.5-fold greater for the intercropped wheat compared to wheat alone (Marschner et al. 1986). Likewise, pigeon pea can grow and yield well in soils with low available P because of its ability to utilize Fe-P (Ae et al. 1990) by release of piscidic acid. Released P may become available for a companion crop. Alternatively, the incorporation of P from unavailable inorganic Fe-P into pigeon pea tissues provides a subsequent crop, grown in rotation, with a more easily accessible form of organic P. Snapp (1998) has identified long duration legumes that have indeterminate growth and a low harvest index as critical partners to improving P sustainability in smallholder agriculture. Such species (soybean, groundnut, pigeon pea, velvet bean) not only release P through acidification and acid phosphatases but also provide P through high quality leaf residue that is incorporated into soil organic matter. Common bean shows potential for enhancing P sustainability in South China (Yan 1998). Genetic variation for organic acid exudation and root branching under low P have been identified in common bean and is being incorporated into selected lines.

6. Synopsis

Nitrogen and P pose a dilemma for agriculture. Although both are required for production of high quality, protein-rich crops, purchase and application of these fertilizer elements are the two most costly inputs for farmers. Their ease of access and overuse in the intensive fanning of developed countries have led to significant environmental degradation. In contrast, in the extensive farming systems prevalent in developing countries the high cost and limited availability of N and P restrict yield and reduce quality. An additional problem is that production of N and P fertilizers depends upon nonrenewable resources and as currently used is not sustainable. Because of their capacity for BNF, legumes are critical to N sustainability through providing a low cost, renewable supply of N that is less prone to leaching and volatilization. Now it is becoming quite clear that legumes have a major role to play in P sustainability. They have several mechanisms for solubilizing unavailable P which results in enhanced P acquisition particularly in low P soils. Incorporation of legumes into inter- and rotational-cropping systems provides a low cost alternative to adding P fertilizer for improved soil fertility.

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THE BRAZILIAN EXPERIENCE WITH THE SOYBEAN (*GLYCINE MAX*) AND COMMON BEAN *(PHASEOLUS VULGARIS)* SYMBIOSES

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1. Introduction

In the tropics, the fragile soil structure, low soil fertility and inappropriate farming technologies can result in low yields. In this context, biological N_2 fixation (BNF) is a critical and key process, since N is a limiting nutrient. Approaches using BNF with the soybean and common bean crops are being used with success in Brazil and should be extrapolated to other countries.

2. Soybean Symbiosis

Soybean was introduced in Brazil in 1882 as *Soja hispida* (*Glycine soja*) and *Soja ochroleuca,* but large scale cultivation began only in the early 1960s. Today, Brazil is the world's second soybean producer, with 13 million hectares planted and almost 31 million metric tons of grain produced. High technology is employed, resulting in an average yield of 2,375 kg ha^{-1}. The high protein content of the grains demands considerable amounts of N. For yields of 2,500 kg ha^{-1}, the crop needs an uptake of 200 kg N ha^{-1}. Since soils are N poor, supplying just 15-30 kg of N $crop^{-1}$ and N fertilizers are very expensive, soybean growth would be economically impracticable without BNF.

The first studies performed in Brazil indicated that the soils were void of soybean bradyrhizobia, hence, during the crop establishment, the seeds were inoculated. Inoculant came initially from USA, but a search for strains adapted to the Brazilian conditions started concomitantly with the expansion of commercial crops, enhancing inoculant production after the mid 1960s. This legume's capacity to satisfy its nutritional N demand via BNF is well documented and today, no N fertilizer is applied to the crop, resulting in an economy estimated in 1 billion dollars per crop season (Vargas, Hungria, 1997). However, the continuous selection of more efficient and competitive strains and of plant genotypes with higher BNF capacity is essential to maintain and increase soybean yields.

2.1. Plant Genotypes

Although soybean breeding programs in Brazil have been performed in soils with an established population of soybean bradyrhizobia, breeders often have neither inoculated the seeds nor evaluated the symbiotic performance, with more attention being paid to resistance to diseases and yield. Recently, differences among cultivars were verified when 152 genotypes recommended for different regions of Brazil were evaluated for BNF capacity, with some of them accumulating up to 4 times more nodule dry weight and 2.5 times more N in tissues. Losses on BNF capacity were verified, e.g., cultivar Davis, a North American genotype introduced in Brazil in the 1960s, showed 30% more nodule number and 34% more N in tissues than a recently released cultivar, BR-16, that had Davis as a parental genotype (Bohrer, Hungria, 1998; Hungria, Bohrer, 1999). In soybean programs, more attention should be given to understanding and evaluating the plant mechanisms controlling and regulating nodule formation and activity, otherwise BNF capacity can be decreased. In this context, a program was started at Embrapa aiming both to obtain cultivars with higher BNF capacity and to study the inheritance of quantitative traits of BNF.

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2.2. Selection of *Bradyrhizobium* Strains

Since bacteria have to meet the increased N demand of more productive cultivars (1,500 kg ha^{-1} in the 1960s and 2,500 kg ha^{-1} today), a strain selection program takes place at Embrapa. The first approach of this program consists of reisolating adapted strains, from areas which have been previously inoculated, looking for variant genotypes with higher competitiveness and BNF capacity. In the 1960s, soybean was cultivated in the South Region of Brazil, but later, a new agricultural frontier was opened, the "Cerrados", an edaphic type of savanna occupying 25% of Brazilian land and characterized by low fertile soils (acidic, with toxic level of aluminum and poor on N and P), dry spells and high temperatures. The first inoculants carried a very competitive strain, SEMIA 566, commercially used in Brazilian inoculants from 1966 to 1978. Now, as the search of new strains was limited by the lack of tolerance to environmental stresses, soybean was sown in areas that had been inoculated with SEMIA 566 about 15 to 25 years before, with no further inoculation. Bacteria were reisolated from large pink nodules and tested individually for N_2 fixation capacity and competitiveness under greenhouse conditions, and, subsequently, for grain yield in several field trials. An efficient isolate, CPAC 15 (= SEMIA 5079), proved superior, increasing yield by up to 750 kg ha^{-1} in comparison with the commercial strains and has been officially recommended since 1992 (Vargas et al., 1992; Vargas, Hungria, 1997). Other efficient, competitive strains were obtained using this approach (Boddey, Hungria, 1997; Hungria et al., 1998). Analyses showed that adaptation to the stressful conditions results in several morphological, physiological and genetic changes. Differences reported included an increase in mucus production, in Hai phenotype and a decrease in the synthesis of indole acetic acid and rhizobitoxine in variant strains, as well as differences on nodulation with cultivar Hill (*Rj₄*) and modifications in DNA profile after amplification by RAPD or by ERIC and REP-PCR (Hungria et al., 1996; Nishi et al., 1996; Boddey, Hungria, 1997). However, the ecological importance of these changes is still unknown.

A second approach in Embrapa's strain selection program tests individual colonies from a pure culture, searching for variant strains showing higher BNF capacity and competitiveness. Success occurred with one very efficient but low competitive strain, CB 1809, sent from Australia in 1966. Testing dozens of individual colonies identified one variant strain, CPAC 7 (=SEMIA 5080), with higher competitiveness leading to increases in nodulation, nodule occupancy and yield in several field experiments. This strain has been officially recommended since 1992 (Vargas et al., 1992; Vargas, Hungria, 1997). Other variants were obtained using this approach and increases of up to 132% in nodule occupancy were obtained in relation to the parental strain. More detailed studies have also identified morphological, physiological and genetic differences between the parental CB 1809 and the variant strains, e.g., on colony morphology, Hai phenotype and Nod factor profile (Hungria et al., 1996, Nishi et al., 1996). Few polymorphisms were detected when parental and variant strains were amplified with short primers (RAPD) or by ERIC and REP-PCR (Santos et al., 1999), thus showing lower genetic diversity than that observed with variant adapted strains.

The rhizobiologists meet every two years and, based on the results from field experiments, decide which strains will be officially recommended for each legume species. Inoculants must carry exclusively the recommended strains that are given without any cost to the industry. Concentration must be of 10^8 cells g^{-1} or ml^{-1} of inoculant, allowing 80,000 cells $seed^{-1}$, but better results have been obtained with 160,000 cells $seed^{-1}$. The inoculants must be absent of contaminants at the 10^{-5} concentration. There are four strains recommended for the soybean crop: SEMIA 587, SEMIA 5019 (=29w), SEMIA 5079 and SEMIA 5080 and inoculants must carry two of them.

2.3. Effects of Reinoculation

Until two years ago, the majority of the inoculants sold in the country were peat based, but the participation of liquid inoculants increased to 40% of the market. However, better results at the field have been obtained with peat based inoculants, probably because the peat gives some physical protection to water and temperature stresses. Furthermore, most seeds are treated with fungicides and the peat could also give some protection against the toxicity by those products. An adhesive, usually a sucrose solution (10%), is essential to stick the peat inoculant to the seeds. Today, there are few first year soybean areas remaining, and most soils show a naturalized population prevailing the most competitive strains used in the past years. Consequently, the introduction of new and more efficient strains can be very difficult and therefore a National net trial has started in 1993 to verify the effects of reinoculation. In 13 trials conducted from 1993-1996 in seven counties, reinoculation increased yield by up to 23% and total N in grains by up to 25%. The best strain combination (SEMIA 587 + SEMIA 5080) allowed average yield increases of 7.8% and 3.8%, in the South and Central Regions, respectively. Although the soils showed a very high naturalized population (10^3 to 10^6 cells g^{-1} of soil), reinoculation increased nodulation and nodule occupancy by the strains carried in the inoculant. In these experiments, addition of N fertilizer from "starter" doses of 10-30 kg of N ha^{-1} to heavier doses of up to 400 kg of N ha^{-1} never produces yield increases. The communication of these results to the farmers increased the reinoculation rate from 28 to 60%.

2.4. Ecology of *Bradyrhizobium*

Recently, modern and primitive soybean genotypes were used as trapper plants for rhizobial strains. Soil samples were collected from sites thousands of kilometers appart, cropped or undisturbed and covered with natural vegetation. First, *Bradyrhizobium* strains were isolated from undisturbed soils far from cropping areas. Morphological, physiological and genetic characterization indicated that some few strains could be indigenous, but most of them were similar to the strains used in commercial inoculants, indicating dispersion of bacteria.

Fast growing strains were also isolated from soybean nodules inoculated with soil dilutions from both cropped and undisturbed areas. In many morphological and physiological properties they resembled *Sinorhizobium fredii* but other analyses, such as ERIC and REP-PCR and profile of fatty acids indicated that the strains showed unique characteristics probably fitting into a new species. Fast growers represented about 20% of the population able to nodulate soybean, but the strains were very poor competitors against *B. japonicum* and *B. elkanii.*

3. Common Bean Symbiosis

Common beans contribute to about 28% of the population's protein consumption and is cropped in an area of 4.12 million hectares, but the yield is low, 710 kg ha^{-1}. Brazilian soils show a very high population of bean rhizobia and therefore the inoculation with superior strains often does not bring any benefit to the plants. Therefore, while 12,379,556 doses of inoculants were sold for the soybean last season, just 121,333 doses were commercialized for the common bean crop. However, a long term search for strains that are genetically stable and adapted to environmentally stressful conditions which frequently occur in these areas was missing. Furthermore, many years of experimentation were lost because the tested strains were genetically unstable (Hungria et al., 1997). With the reports that genetic stability of *Rhizobium tropici* was superior to that of the other bean rhizobia species, a selection program has started searching for strains belonging to this species. First, several isolates from the State of Parana, able to nodulate both common bean and *Leucaena* spp. plants were tested to identify superior genotypes. The most promising strain identified, PRF 81 (=SEMIA 4080), showed high rates of N_2 fixation, was competitive and tolerant to high temperatures. The good performance was confirmed in four field experiments, where inoculation with PRF 81 resulted in yields of up to 3,200 kg ha^{-1} and gains of 900 kg of grains ha^{-1}, compared to the indigenous population (10^5 cells g^{-1} of soil). The positive results obtained in other field experiments lead to the official recommendation of PRF 81 since 1998 (Hungria et al., 1999), together with *R. tropici* type IIB CIAT 899. Characterization of PRF 81 by growth capacity in different media, tolerance to acidity and high temperature, Nod factor profile, profiles of RAPD and ERIC and REP-PCR, PCR product obtained by the amplification with Y1-Y2 primers and profile of fatty acids indicated that the strain has mixed characteristics between *R. tropici* type IIA and IIB. The results obtained with PRF 81 encourage the identification of new competitive and efficient *R. tropici* strains for tropical areas, reverting the actual trend of low yield and depletion of soil N.

4. Soil Management

Agricultural practices as crop rotation with some legumes and the no-tillage system can improve BNF and yields. For example, the no-tillage system preserves fragile soils against degradation and results in lower soil temperatures and higher moisture content, favouring the BNF process. Consequently, a comparison of the no-tillage and conventional tillage systems showed that in the first the number of *Rhizobium* and *Bradyrhizobium* cells, the amount of *nod*-gene inducing compounds accumulated in the soil and the genetic diversity were higher. Nodulation, N_2 fixation rates and yield were also higher under the no-till.

5. Conclusions

Soybean and common bean are cultivated on more than 17 million hectares in Brazil. Soybean is the main cash crop and relies exclusively on BNF to supply the plant N demands. However, to maintain yields of more productive cultivars, a search for efficient, competitive and stress tolerant strains continues. Common bean is the most important legume used for food and inoculation with an efficient, competitive *Rhizobium tropici* strain increases in nodulation, nodule occupancy and yield. An adequate soil management is essential to guarantee the benefits of BNF with both crops.

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QUANTIFICATION OF BIOLOGICAL NITROGEN FIXATION IN AGRICULTURAL SYSTEMS

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1. Introduction

That symbiotic N_2 fixation by legumes plays a key role in global crop production is irrefutable. The 163 million ha of legume oilseeds (soybean and groundnut) and pulses grown each year, legume components of the 200 million ha under temporary pastures or fodder crops, and the 10-12 million ha of perennial legume cover-crops in rubber and oil-palm plantations all contribute fixed N to production systems. However, while it is recognised that BNF provides a renewable N source for agriculture, effective management of BNF ultimately relies on an ability to accurately measure it.

2. Measurement of BNF

Many early determinations of BNF were based on the N yield of a legume. This approach overestimated the amounts of N_2 fixed as it erroneously assumed that all the N present originated from N_2 fixation. The acetylene reduction assay attempted to redress this situation by relating indirect measures of nitrogenase activity by detached nodules or nodulated roots to rates of N_2 fixation. However, errors inherent in the methodology and variable relationships between ethylene production and N_2 fixation made this approach impractical for field studies. Currently-used methods separate legume N into two fractions, i.e. N originating from soil N or atmospheric N_2. The basic N-difference procedure compares the amount of N accumulated by a legume with that of a nearby non-legume and attributes the difference to BNF. Accuracy can be further increased, at least in theory, with the ^{15}N enrichment and natural ^{15}N abundance methods. With these techniques, the ^{15}N content of the legume is compared to non N_2-fixing reference plants which are used to sample the ^{15}N composition of plant-available soil N. Legume and reference plants do not have to use the same amounts of soil N; however, it is assumed that they use soil N with the same ^{15}N enrichment. The ^{15}N value of a legume will be the same as the reference if no N_2 fixation occurs and will approach the ^{15}N composition of atmospheric N_2 as the legume's reliance upon BNF increases. The ^{15}N-based methodologies provide estimates of proportion of legume N derived from N_2 fixation, usually expressed as %Ndfa or %Pfix, and must be combined with total plant or crop N values to determine the amounts of N fixed.

The xylem ureide method also provides estimates of %Ndfa which are derived from colorimetric analyses of the xylem sap (and stem extracts) for ureide, amino and nitrate N. The method is a point-of-time assay and must be repeated during the legume's growth cycle to obtain a seasonal profile of %Ndfa, which is then combined with plant N estimates to determine total N fixed. The choice of method for a particular laboratory will depend on resources, species of legume studied and the overall objective, e.g. whether an estimate of %Ndfa is required or whether the objective is to determine total N fixed. Key reviews of the various techniques include: Chalk (1985); Shearer and Kohl (1986); Ledgard and Peoples (1988); Peoples and Herridge (1990); Danso et al. (1993); Herridge and Danso (1995); Unkovich et al. (1997).

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3. The unrecognised contribution of below-ground N

In the past it was generally assumed that N in the nodulated roots of legumes represented at most 10-15% of total legume N. It seemed reasonable, therefore, to ignore root N and to simply use shoot N as a surrogate measure of whole plant N. Recent research, however, has sought more accurate estimates of below-ground N of legumes using ^{15}N shoot-labelling techniques. Results indicated that N either associated with or derived from nodulated roots can represent 40-50% of the total plant N of pasture legumes (Zebarth et al. 1991; McNeill et al. 1997), and between 27-40% for crop legumes (Russell and Fillery 1996; Rochester et all 998). Therefore, total inputs of fixed N could be in the order of 50-100% greater than that determined from shoot-based measurements.

The impact of accurately estimating and accounting for below-ground N when budgeting the rotational benefits of legumes is illustrated in Figures 1 and 2, using data of commercial faba bean crops in Australia. An additional 6-210 kg N ha^{-1} (average of 105 kg N ha^{-1}) was calculated to be associated with roots, i.e. additional to that fixed into the shoots (Fig.l). Grain yields by the 35 commercial crops averaged 2 t ha^{-1} which removed 64 kg N ha^{-1}. Determinations of the residual fixed N remaining after grain harvest suggested an average net input of fixed N of +8 kg N ha^{-1} when shoot-based measures of BNF were used for the calculations. However, average inputs of fixed N were increased to +113 kg N ha^{-1} when estimates of below-ground N were included (Fig.2). Clearly below-ground N should be considered in all future studies of N_2-fixing legumes and their contributions to agricultural systems.

4. BNF in agricultural systems

Published estimates of BNF by crop legumes are in the range 100-300 kg N ha^{-1} (Peoples et al. 1995b). These estimates have largely been derived from research trials where specific treatments may have been imposed to generate differences in %Ndfa and legume growth in order to study factors affecting BNF. However, these data may have little relevance to farmers' legume crops and pastures. Procedures, such as the natural ^{15}N abundance and the ureide methods, have now been developed to the point that on-farm measures of legume N_2 fixation can be conducted with confidence.

Examples of the use of the natural ^{15}N abundance method to provide on-farm estimates of BNF in rainfed cropping systems of South Asia are presented in Table 1 and Figure 3. The levels of $\delta^{15}N$ (measure of the ^{15}N enrichment in parts per thousand) detected in farmers' legume crops were compared with either non-legume weeds or intercropped cereal or oilseed reference plants growing in the same field (Fig. 3). Although the $\delta^{15}N$ values of the reference species were somewhat lower than commonly found in most other farming systems, the $\delta^{15}N$ values of the legumes were usually 1-3‰ below the reference indicating measurable 'dilution' of soil ^{15}N by atmospheric N_2.

The data from Pakistan indicated that there can be large differences in the distribution of Ndfa values for different legume species. With lentil and chickpea, the majority of farmers' crops exhibited %Ndfa between 60-90% (Table 1). Mung bean on the other hand, showed a wider distribution of Ndfa values, from 0-100%, with the majority of crops around 50% (Table 1). Similar variations in %Ndfa between different legume species have also been observed in Nepalese crops. However, when amounts of N_2 fixed have been calculated, crop growth rather than %Ndfa was generally the dominant factor determining BNF. Most crops fixed around 20 kg of shoot N (equivalent to 30 kg total N fixed with allowance for below-ground N) for every tonne DM accumulated.

Figure 1. The amounts of N_2 fixed by commercial faba bean crops in Australia where estimates of fixed N associated with the nodulated roots were either (a) omitted, or (b) included in the calculations (after Rochester et al. 1998).

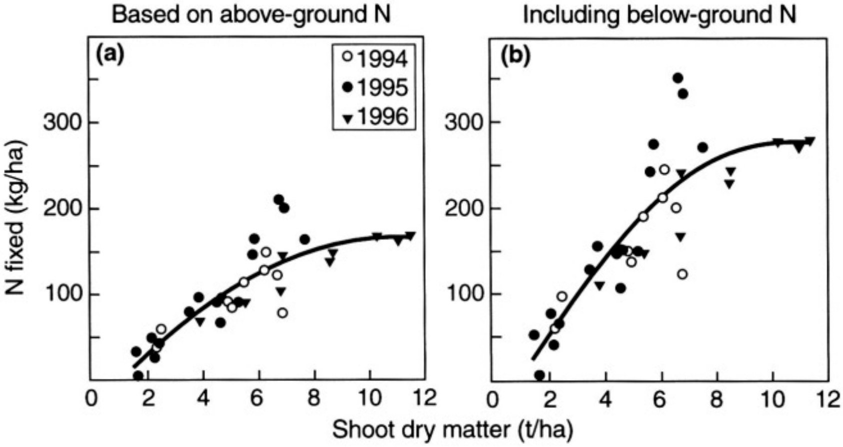
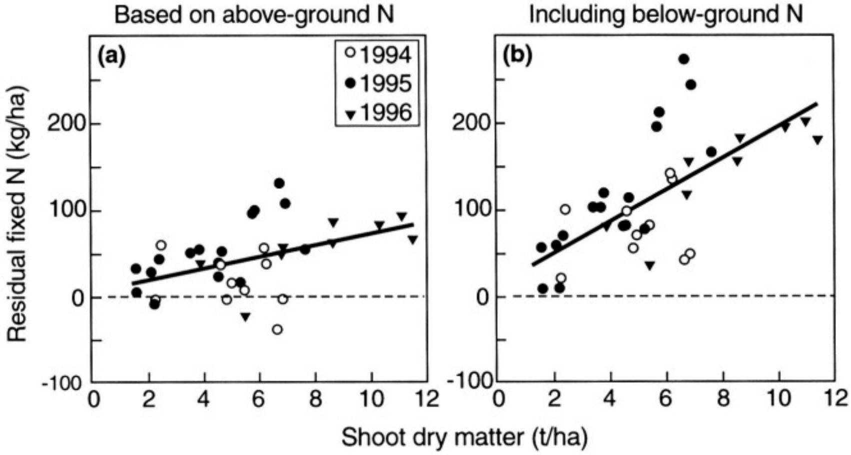


Figure 2. Residual fixed N remaining in the soil after grain harvest of commercial faba bean crops in Australia where estimates of fixed N associated with the nodulated roots were either (a) omitted, or (b) included in the calculations (after Rochester et al. 1998).



On-farm surveys of BNF have also been conducted in other geographical regions. Collectively the results indicate that the potential for BNF can differ between legumes and countries, but also suggest many commonalties (Table 2).

Figure 3. Levels of natural ^{15}N abundance ($\delta^{15}N$) in legumes and in-crop weeds in farmers' fields in different regions of northern Pakistan (source: Rupela et al. 1997).

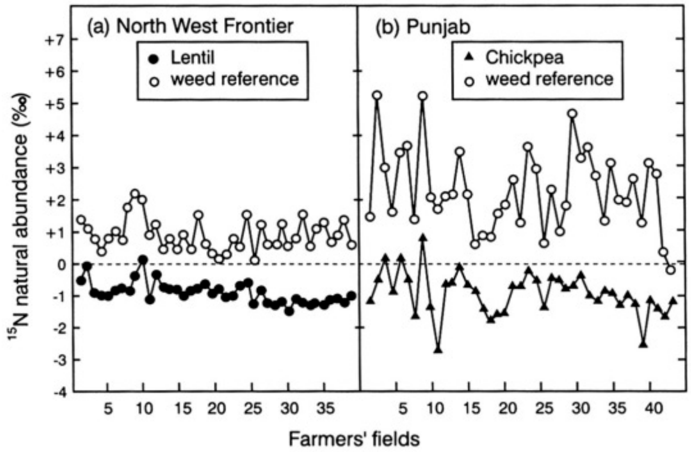


Table 1. Distributions of %Ndfa of farmers' crops of legumes in northern Pakistan (source: Rupela et al. 1997).

Species	Number of crops surveyed	Level of %Ndfa 0-20	 21-40	 41-60	 61-80	 81-100
		% crops in each category				
Lentil	40	0	0	8	50	42
Chickpea	86	0	3	8	43	45
Mung bean	40	15	15	35	25	10

Although similar ranges of %Ndfa to those depicted in Table 1 were generally observed in each location, the summer legumes, mung bean and soybean, tended to have a lower overall dependence on N_2 fixation (47-75%) than the winter pulses such as chickpea, lentil, lupin and faba bean (65-79%, Table 2). The lower values may be related to a lower crop demand for N in some situations, but is more likely because of suppression of BNF by soil nitrate, mineralised during the warm and often moist summer season. In grazed pastures on the other hand, the competition for mineral N between legumes and the often more vigorous companion grasses and broadleaf weeds in the sward results in low levels of nitrate and minimal suppression of BNF (Peoples and Baldock 2000). Thus, %Ndfa by pasture legumes were consistently high (64-76%). The slightly lower %Ndfa values for the perennial pastures, containing species such as alfalfa and white clover, most likely reflected those species' ability to scavenge greater amounts of soil mineral N from deeper in the soil profile than the annual species.

In addition to quantifying inputs of fixed N as part of overall N management, such information can be used to identify practises which may either enhance or depress BNF on farm. The principal factors regulating BNF (Table 3) can generally be summarised in terms of environmental or management constraints to crop growth (basic agronomy, nutrition, water supply, diseases and pests), and local practises that either limit the presence of effective rhizobia (no inoculation, poor inoculant quality),

Table 2. Summary of surveys of BNF of legumes in farmers' fields in Pakistan, Syria, Nepal, Thailand, South Africa and Australia, using natural ^{15}N abundance and xylem ureide methods.

Country and legume	Number of fields	Mean Ndfa (%)	Total N fixed[a] (kg N ha^{-1})
Winter pulses			
Pakistan	126	78	79
Nepal	27	79	78
Syria	46	67	-
Australia	90	65	170
Summer legumes			
Pakistan	63	47	42
Nepal	34	57	41
Thailand	13	75	78
South Africa	14	58	-
Australia	33	53	267
Annual pastures			
Australia	293	76	-
Perennial pastures			
Australia	110	64	-

[a] Collated from Unkovich et al (1994), Peoples et al (1995a), Rupela et al. (1997); Rochester et al. (1998), Schwenke et al. (1998), Peoples and Baldock (2000), and unpublished data. Total N_2 fixed was calculated assuming below-ground N represents 33% of total plant N in crops and 50% in pastures.

Table 3. Factors regulating BNF in farmers' fields

Country	System	BNF regulated by-		Primary factors
		Yield	Soil nitrate	
Pakistan	Winter crop	+++		Rainfall, basic agronomy, weed control
	Summer crop	+++	+++	Soybean: fertilizer N/ no inoculation Mung bean: insects and disease
Nepal	Winter crop	+++		Rainfall, basic agronomy, nutrition
	Summer crop	+++	+++	Basic agronomy, currently mineralised nitrate-N
Syria	Winter crop	+++	++	Soil nutrients, insects
Thailand	Summer crop	+++		Inadequate P
South Africa	Summer crop	++	++	Effective inoculation, crop nutrition, rotation and water availability
Australia	Winter crop	+++	+++	Rainfall. Faba bean less sensitive to soil nitrate than chickpea.
	Summer crop	+	+++	Tillage and fallow length
	Pasture	+++	+	Linked to total soil N, P & K nutrition, soil pH, rhizobial numbers, nematodes, rainfall

or directly affect soil nitrate fertility (excessive tillage, extended fallows, fertilizer N, rotations). These same factors appear to be common across species, countries and regions. We have used the natural ^{15}N abundance and the ureide techniques exclusively in these studies and are confident that the estimates will provide a valuable data base to be used to enhance the efficiency of the process of BNF and of the use of the fixed N in the particular agricultural systems.

5. Summary

^{15}N natural abundance method and the ureide assay have now been developed to the point that on-farm measures of legume N_2 fixation can be conducted with confidence. Data from diverse systems (pastures, pulses, or legume oilseeds in areas of Australia, Asia, the Middle East and Africa) show clearly that environmental and management constraints to legume growth (basic agronomy, nutrition, water supply, diseases and pests) are the major regulators of N_2 fixation, although practices that either limit the presence of effective rhizobia in the soil (no inoculation, poor inoculant quality), or enhance soil nitrate levels (excessive tillage, extended fallows, fertilizer N, rotations) can also be critical. In the past, the amounts of N_2 fixed by legumes have been derived from shoot-based determinations of plant N. However, recent applications of ^{15}N shoot-feeding techniques to crop and pasture legumes suggest that a major portion (30-50%) of the total plant N is associated with the nodulated roots. Therefore, it is critical that estimates of below-ground N are included when biological nitrogen fixation (BNF) is quantified. Otherwise, the contributions of legumes to the N economies of agricultural systems may be substantially underestimated.

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PUTTING LEGUME N_2-FIXATION TO WORK IN CROPPING SYSTEMS OF SOUTHERN AFRICA

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1. Introduction

Much has been written on the importance of legume N_2-fixation for agriculture in developing countries but what role does it play on farms of the rural poor in Africa? Recent initiatives have highlighted the decline in soil fertility in many African soils, and have attracted much donor attention to address the problems of impoverished soils and how the fertility of these can be restored (Buresh et al., 1997). Much of the emphasis for the renewed focus on soil fertility declines in African farming systems comes from nutrient balance studies (e.g. Smaling et al., 1993; Stoorvogel et al., 1993; Smaling, Nandwa, 1997). These studies have examined nutrient budgets at a number of scales, from the farm to the subcontinent and concluded that rates of decline in soil N stocks vary from 22-112 kg N/ha/yr (Smaling, Nandwa, 1997). Information from studies of N_2-fixation under farmers' field conditions on which nutrient balance studies can be based is very limited. In most of the nutrient balance studies, rates of N_2-fixation by legumes have been taken to be 50%, though without a firm basis of on-farm measurements of N_2-fixation.

The current and potential roles of grain, forage and tree legumes in the restoration and maintenance of soil fertility in Africa were discussed in a general review by Giller et al., (1997). Here we evaluate the potential roles of legumes in the maintenance of soil fertility in Malawi and Zimbabwe, using results from a wide range of studies on farmers' fields in which rates of N_2-fixation have been estimated using the ^{15}N natural abundance method (Shearer, Kohl, 1986; Peoples et al., 1989). Research summarised here was conducted under the umbrella of the Soil Fertility Network for Maize-based Cropping Systems in Southern Africa. This is a coordinated network of soil scientists, agronomists, extension workers and economists striving to identify practical options to address the acute problems of poor soil fertility and to assist smallholder farmers in improving their livelihoods though enhanced agricultural productivity.

2. Materials and Methods

Given limitations of space it is not possible to give detailed methods for most of the agronomic information and readers are referred to primary sources where possible. N_2-fixation estimates made using the ^{15}N natural abundance method (see Peoples and Herridge, this volume). Nitrogen contents and ^{15}N- natural abundance of samples were directly analysed in dried, ground tissue samples using a Europa Scientific Roboprep automated C/N analyser coupled to a 20:20 mass spectrometer. The B values used were determined at Wye College or taken from the literature. In several cases the non-fixing reference plants had $\delta^{15}N$ values of 2 ‰ or less. However, where sampling was highly replicated, consistent differences between non-fixing reference plants and nodulated legumes were

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found giving some confidence in the measurements. This is similar to experience reported from wide-ranging studies in Asia (Peoples et al., 1997). Other problems encountered using the natural abundance method were different enrichments of mineral fertilizers applied (ammonium nitrate 2.17 $\delta^{15}N$, urea -3.39 $\delta^{15}N$, compound fertilizer 3.17 $\delta^{15}N$), highly variable natural abundance in reference trees when making measurements associated with *Faidherbia albida.* In particular mango *(Mangifera indicd)* had unexpecedly low $\delta^{15}N$ values compared with other non-fixing trees such as *Adansonia digitata, Kigelia africana* and *Piliostigma thoningii.*

3. Increasing Inputs from Legume N_2-fixation in Maize-based Cropping Systems

In southern Malawi the area of land available to most households is 0.4 ha or less and 90% of land is cropped to maize. Population pressure in most smallholder communities in Zimbabwe is less acute, but farms are often only 1.5 ha and the sandy granitic soils are highly infertile. Use of agricultural inputs like mineral fertilizers is limited due to lack of financial resources and supply. In both countries, N is the major limiting nutrient for crop production indicating the major role that N_2-fixation by legumes plays in increasing agricultural productivity. The major constraints to N_2-fixation are poor productivity of the legumes within the systems and the limited area of land planted to legumes at any time. The uni-modal rainfall pattern and long dry season of 7-8 months in southern Africa means that only a single crop can be grown each year.

3.1. Grain Legume Rotations

The major grain legume in Zimbabwe and parts of Malawi is groundnut (*Arachis hypogaea*) but yields are poor (0.2-0.5 t/ha) due to a combination of poor seed quality and varieties, low population densities and lack of basal fertilizer use (Shumba, 1983). The poor groundnut yields and high labour requirements render groundnut-maize rotations unprofitable compared with continuous maize cultivation (Waddington, Karigwindi, submitted). Indeed, although substantial residual benefits of groundnut on maize yields on poor soils on-station have been demonstrated, maize yields were increased by only 0.3 t/ha on smallholder farms due to addition of 20 kgN/ha by the groundnut crop (Jeranyama et al., submitted). In Malawi, new varieties of common bean (*Phaseolns vulgaris*), specifically bred for poor soil fertility conditions found on smallholder farms, yield up to 1.5 t/ha whereas local varieties generally yield less than 0.5 t/ha due to a combination of better disease resistance and improved environmental adaptation (R. Chirwa, pers. comm.). Amounts of N_2-fixation in these new bean varieties have not been determined but are certain to be part of the improved yield potential, although residual benefits of beans are generally limited (Giller, Wilson, 1991). Cowpea (*Vigna unguiculatd*) is commonly grown on small areas by farmers though insect damage and poor storage qualities of the seed limit its productivity and hence inputs from N_2-fixation.

Until recently it was believed that soyabean could not be grown widely in the sandy infertile soils of Zimbabwe. At a workshop held to solicit views of stakeholders concerning the potential of soyabean as a smallholder crop, and the potential benefits for soil fertility, a Soyabean Promotion Task Force was established and charged with the responsibility of promoting soyabean in the smallholder farming sector of Zimbabwe (Mpepereki et al., 1996). The initial emphasis was on the use of promiscuously nodulating soyabean varieties which are able to give good yields with limited inputs and which have greater soil fertility benefits than specifically-nodulating varieties (Kasasa et al., 1998, Mpepereki et al., 1999). Both types of soyabean varieties fixed around 80% of their N in on-farm trials and the amounts of N_2-fixation and of N in stover produced by the promiscuously-nodulating varieties were greater than the specific varieties (Table 1). Clear benefits in maize yields from rotation with soyabean were found in all experiments but productivity was generally much less on farmers' fields than the potential shown in on-station experiments (Table 1). The increases in maize yields were greatest when soyabean stover was incorporated but substantial benefits were found even when it was removed from fields to mimic grazing by cattle.

Table 1. Residual benefits to maize of promiscuously-nodulating soyabean varieties 'Magoye' and 'Local' and specifically-nodulating varieties 'Roan' and 'Nyala' all grown with inoculation on the University of Zimbabwe farm and on a farmer's field in Zimbabwe in 1997/98. No N fertilizer was applied to maize.

Variety	Stover N added (kg/há)	Maize grain yields (t/ha)	
		- soyabean stover	+ soyabean stover
University Farm			
Magoye	153	3.0	4.2
Local	143	3.5	4.5
Roan	55	3.7	4.2
Nyala	32	3.4	4.0
Maize after maize		1.8	
SED		0.3	
Farmer's field (Tapera)			
Magoye	50	1.1	1.5
Local	47	1.0	1.3
Roan	29	0.8	1.0
Nyala	28	0.7	1.1
Maize after maize		0.4	
SED		0.19	

In the pilot soyabean promotion phase (1996/97) inputs such as lime, seed and fertilizers were donated by local agro-industries and the demonstration/extension activities aroused intense interest from farmers. The number of cooperating farmers rose from 50 in 1996/97 to more than 6000 in the 1998/99 season. The majority of soyabeans currently being grown by smallholders are specifically-nodulating varieties inoculated with rhizobial inoculants which are produced locally as seed of the promiscuously-nodulating variety 'Magoye' is available in only limited quantities. Farmers have been encouraged to retain their own seed for the next season which allows them to expand their area cropped to soyabean. This has been truly demand driven: farmers have paid for all the inputs including seed, inoculants and the small amount of fertilizer required. The project has handled the sale of produce to oil processing companies on behalf of the farmers. This has allowed amounts ranging from as little as 7 kg to 3,000 kg to be purchased from individual farmers and has not discriminated against the poorer participating farmers. Activities organised have also included field tours for farmers and extension agents and cooking demonstrations to promote domestic consumption of soyabean. Once farmers realised the nutritional benefits of soyabean and its good storage qualities they have often opted to retain a substantial proportion of their produce for home consumption (see Mpepereki et al., this volume) and a number of NGO's are actively involved to promote the crop.

A traditional intercrop in southern Malawi is to grow maize with pigeonpea. Growth of pigeonpea is sufficiently slow that the intercrop maize can be sown with the same maize plant population as the sole crop with no yield penalty to maize. With introduced *Fusarium* wilt resistant pigeonpea varieties, pigeonpea fixes over 80% of its N when intercropped with maize (Table 2) and up to 75 kg N/ha can be added from fallen leaves when pigeonpea grows well (Sakala, 1998). The fallen leaves induce net N immobilization for up to 2 months as they decompose due to their relatively small N (1.8%N) and high lignin content of the leaves, which means that only the subsequent crops benefit from the additions to the soil (Sakala et al., 2000). Recent production of organic pigeonpea has promise for improving farmers opportunities for earning cash income through growing what has generally been looked on as a food crop. In Zimbabwe pigeonpea is not grown widely and although in testing on farm it has shown potential its yields and therefore the amounts of N_2-fixation have been highly variable on smallholder farms.

Table 2. N_2-fixation in pigeonpea measured using the natural abundance method in Malawi and Zimbabwe

	%N from N_2-fixation	N_2-fixed (kg/ha)
Malawi (3 sites)		
Sole crop	42-64	13-146
Intercrop	82-85	48-163
Zimbabwe (10 sites)		
Sole crop	82-85	2-45

3.2. Green Manure and Forage Legumes

Green manure legumes such as sunnhemp (*Crotalaria juncea*) and velvet bean (*Mucuna pruriens* var. *utilis*) were widely used to maintain soil fertility on commercial farms in southern Africa before mineral fertilizers became widely available. There has been limited use of green manures by smallholder fanners despite the poor fertility of their lands and the current economic restrictions on mineral fertilizer use. *Mucuna* is the most productive of the green manures although in the most degraded soils in Zimbabwe none of the legumes which have been tested grew well, even when basal P fertilizers were supplied. A range of green manure species fixed 37-78% of their N on farmers' fields in Malawi amounting to inputs up to 75 kg N/ha. In Malawi, farmers have little land and are unable to devote land for green manures for soil improvement although *Mucuna* can produce up to 2 t/ha grain on poor soils where maize barely yields at all. Although the seed of *Mucuna* contains large concentrations of L-dopa and requires long cooking times before eaten, it is used as a food in Malawi which makes it of more interest to farmers than other legumes. *Tephrosia vogelii* shows great potential as a green manure legume for undersowing as it grows slowly initially and therefore, similar to pigeonpea affords little competition to the maize crop. This legume seeds prolifically, is known to farmers as a fish poison and is attracting their interest for soil fertility improvement.

Communal grazing in Zimbabwe means that there is little incentive for individual farmers to invest in improving forage for their cattle as few fields are fenced. However some farmers groups, which focus on dairying or fattening cattle for beef, are interested in enclosing some land to grow fodder and are beginning to experiment with forage legumes. Of the forage legumes tested, siratro (*Macroptilium atropurpureum*) establishes well on the sandy soil and grows prolifically. A programme of legume screening has been established to evaluate new legumes which may have potential for fodder and soil fertility in difficult soils.

3.3. Agroforestry - Fitting Tree Legumes into Cropping Systems

In the early 1990's there was a strong push to extend the use of alley cropping among smallholder fanners in Malawi as a potential solution to their problems of soil fertility and needs for soil conservation and fuelwood. Unfortunately due to problems of lack of available seed for *Gliricidia sepium* and the advent of pest problems with *Leucaena leucocephala* (both psyllids and termites), the main species supplied to thousands of farmers was the non-nodulating legume *Senna spectabilis*. Although *Senna* had shown promise in alley cropping experiments on good research station soils where it could effectively capture N which was returned in prunings for maize, on soils of poor fertility it competed strongly with maize (Itimu, 1997), and fanners realised no soil fertility benefits from its introduction. In alley cropping experiments where N_2-fixation in *Gliricidia* was examined using *Senna* as a reference plant no consistent differences in $\delta^{15}N$ were found between the two species. This may have been due to limited N_2-fixation, although nodules were frequently found on *Gliricidia* roots and indicates that *Senna* was taking much of its N from *Gliricidia* plots.

Maintenance of parklands of large trees of *Faidherbia albida* in cultivated fields is a traditional practice on many smallholder farms on alluvial soils in the lakeshore region of Malawi. *Faidherbia* exhibits a 'reverse phenology' in that it comes into leaf only at the end of the rainy season and loses all of it leaves when the rains begin. This affords little competition for light with crops growing under the canopy and maize yields are much higher under *Faidherbia* trees than in the open fields (Saka et al., 1994). Although trees are large (23-27 m tall) with individual canopies covering 380 m^2 on average, all of the trees were aged to be only 21-25 years old, supporting conclusions that *Acacia* trees are often much younger than commonly assumed (Gourlay, 1995). Rooting studies, both by excavation of pits roughly 2 m deep and 8 m in diameter around the base of trees and by examining root profiles on eroding river courses have demonstrated that *Faidherbia* does not have extensive rooting in the surface soil horizons (Phombeya, 1999). Although roots branch horizontally from the base of the trunk at 0.5-1 m below the soil surface, they turn vertically down as 'sinker' roots within 3 m from the trunk. Bore wells sleeved with PVC pipe were installed with a hand-operated drilling rig up to 17m depth to allow determination of the depth and mineral N concentration in the ground water. Trees accessed water at 5 to 15m depth during the dry season depending on the location and the concentrations of mineral N in the water ranged from 3-24 mg/1. The proportion of N from N_2-fixation in large *Faidherbia* trees ranged from 24-43% across sites suggesting that the remainder of the N was obtained from the groundwater. Leaf and pod fall contributed a total of 130 kg N/ha to the soil under the canopies, with leaves accounting for roughly two-thirds of this N. A survey across fields with *Faidherbia* trees of different ages revealed that benefits to soil fertility are realised within 10 years of planting, indicating that a programme of investment in progressive planting of such trees in farmers fields is warranted. Current planting densities of the trees (ca. 28 per ha) appear to be close to optimal.

4. Conclusions - The Major Factors Limiting Benefits from N_2-fixation

In most reviews on factors limiting inputs from N_2-fixation, environmental constraints such as drought, poor soil P availability and soil acidity are cited. The major overriding constraint to N_2-fixation in Malawi and Zimbabwe is the small proportion of land cropped with legumes. Where legumes are grown, poor yields and limited inputs from N_2-fixation are due to poor legume varieties lacking disease resistance and poor seed quality in addition to environmental limitations. The largest immediate gains from N_2-fixation come from inclusion of productive legumes in the cropping systems. Farmer's needs for food and income coupled with small farm sizes due to population pressure necessitate ways of fitting more legumes into the system without reducing maize yields. The approaches most attractive to farmers are grain legumes which have both a food and cash value, although some approaches using forage, green manure and tree legumes for soil improvement show promise. However, legume N_2-fixation should be viewed as a complementary source of N rather than a substitute for mineral N fertilizers. An important way in which legumes such as soyabean are contributing to the management of soil fertility is by providing cash which farmers invest to purchase mineral fertilizers for maize.

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MEASURING NITROGEN FIXATION BY TREES

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The application of any technique for quantifying BNF contributions in natural ecosystems, or for woody perennials in agroforestry or plantations, poses special problems. One of the most important constraints to estimating the total BNF input on an annual per hectare basis lies in the estimation of total annual dry matter accumulation (i.e. net primary productivity, NPP). In agroforestry systems it may sometimes be possible to harvest entire trees or shrubs and calculate aerial NPP (and total N accumulation) from the addition of the annual increase in biomass to annual leaf/branch production. However, in mature plantations and natural forests or woodlands, this is rarely possible, and annual growth increments can only be calculated by applying allometric regression equations based on trunk diameter (Brown, 1997). The other principal component of aerial NPP is the litter fall (Stocker et al., 1995). These measurements in natural forests require both intensive and repeated sampling and discussion of the problems involved in estimating NPP of forests, or even tree plantations, is beyond the scope of this paper.

Other difficulties in studies with trees and shrubs which may limit the accuracy of any estimate of N_2 fixation include:

(i) The long-term, perennial nature of growth and the seasonal or year-to-year changes in patterns of N assimilation (e.g. Ladha et al. 1993; Peoples et al. 1996),
(ii) Large plant-to-plant variation in growth and nodulation potential which typically occur even within a single genotype or provenance used for agroforestry,
(iii) Diversity in occurrence and the high degree of variability in distribution of N_2-fixing species, owing to short distance gradients in soil water and nutrient availability, and resource competition in natural ecosystems.

However, in addition to these general constraints, there can be specific problems associated with procedures used to quantify BNF. For example, the acetylene reduction technique has been widely used to study BNF in temperate and tropical forests and agroforestry systems yet it has inherent limitations which make it unsuitable for quantifying fixed N inputs in field studies (Witty and Minchin, 1988). These include the need to recover all the nodules on a plant for assay and the requirement to undertake a series of measurements to cover diurnal, daily and seasonal changes in acetylene reduction which make this approach impractical. In addition, it is also essential to calibrate rates of ethylene production with actual rates of N_2 fixed.

Likewise, to perform a total N balance on trees in the field requires an immense amount of soil and plant sampling, over a period of several years. With exhaustive sampling the difference in N accumulation between the "N_2-fixing" trees and neighbouring non-N_2-fixing species may help to indicate BNF inputs, but as trees vary widely in their capacity to extract N from the soil, no reliable BNF estimates can be obtained using this technique in the field (Ladha et al. 1993). Results using xylem sap-based procedures have been encouraging (Herridge et al. 1996; Peoples et al. 1996); however, this approach is not widely applicable to the most important agroforestry species as they do not produce ureides as a product of N_2 fixation. The utilisation of ^{15}N isotope techniques

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theoretically allows the separate identification of N derived from the soil and from the air. The use of ^{15}N-labelled N_2 gas is impracticable in the field as it would require a gas-tight enclosure of the entire root system. The use of the (^{15}N enriched) ^{15}N dilution technique ideally requires that the soil N is labelled uniformly throughout the rooting zone, and preferably remains approximately stable with time over the period of the study - again this is extremely difficult to achieve in the field for woody perennials.

The technique which holds most promise to quantify the contributions of N_2 fixation to trees in the field is the so-called "^{15}N natural abundance" approach which exploits naturally-occurring differences in ^{15}N abundance between plant-available N sources in the soil and that of gaseous N_2 in the atmosphere. It relies on the commonly-observed phenomenon that soil mineral N (NO_3^- and NH_4^+) is usually slightly naturally enriched (or less frequently, depleted) in the heavy isotope of N, ^{15}N, compared to atmospheric N_2 (Shearer and Kohl, 1986). To calculate the proportion of the plant N derived from atmospheric N_2 (%Ndfa) this technique requires that both the ^{15}N natural abundance of the N derived from BNF and that derived from the soil by the target N_2-fixing species be determined. It is then assumed that the ^{15}N abundance of the N_2-fixing species reflects the relative contributions of the N derived from these two sources. The ^{15}N abundance of the N derived from BNF (*B*) can vary with micro-symbiont, plant species/provenance and growth stage, all of which create considerable difficulties for its precise evaluation. If the %Ndfa is large and the ^{15}N abundance of the N acquired from other sources is not several δ^{15}N units higher or lower than *B*, then this can be a major source of error. Further, more serious difficulties can arise in determining the ^{15}N abundance of the N derived from soil (and plant litter etc.) by the target plant as it is usually impossible to predict which, if any, non-N_2-fixing reference species will obtain N from the same N sources in the same proportions with the same temporal and spatial patterns as the N_2-fixing perennial. The compromise solution is to evaluate the ^{15}N abundance of a diverse range of neighbouring non-N_2-fixing plants and to compare these values with that of the N_2-fixing species and the estimate of *B*. Only then can it be determined whether the contribution of BNF to the target species can be quantified with any degree of confidence.

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BENEFIT OF LEGUME-FIXED N IN CROP ROTATIONS UNDER ZERO-TILLAGE

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1. Introduction

Crops were planted without tillage by ancient civilisations but, later, tillage systems spread throughout the world as they were considered the best strategy to control weeds and produce a seed bed. Today, farmers mainly from Latin America and United States are adopting no-tillage because of its economic advantages compared to traditional tillage, and this has been facilitated by the development of planting machinery and a wider spectrum of more selective herbicides (Derpsch, 1998). In Brazil, the area under no tillage increased from 1 million ha in 1990 to nearly 12 million ha in 1999. For no-tillage adoption to be successful it is necessary to keep constant soil mulch and to have suitable crop rotations to control pests and diseases (Gassen and Gassen, 1996). In order to increase economic benefits, the amount and quality of biomass are also considered in the choice of crops used in the rotation. Biological nitrogen fixation represents a free source of N and any legume is considered to introduce N through crop residues. Soybean is the most common legume planted in Brazil and is the main legume growing in regions such as the Cerrado, where rains limit agricultural exploration to a few months. The objective of this study was to evaluate the amount of N that legumes can fix in the plant-soil system.

2. Material and Methods

Most of the data presented in this paper came from an area located at the Soybean Research Centre of Embrapa, Londrina, Parana. The mean annual rainfall of 1800 mm falls predominantly between October and April but is still enough for agriculture in the remaining months. Monthly temperatures oscillate from 28°C in the wetter period to around 12°C during autumn and winter months (May to August). The soil of the region is a dusky-red latosol that extends through north of Paraná to the south of the states of São Paulo and Mato Grosso do Sul. The area has been under agriculture for many years but for the three years before the implantation of this experiment it was under no-tillage. Three crop sequences under conventional and no tillage were studied: soybean-wheat-soybean; soybean-lupin-maize; maize-oat-soybean. A further study was conducted in the Cerrado (edaphic savanna) area located near Uberlândia, Minas Gerais, which was also under no-tillage for the previous 3 years. Annual rainfall is around 1600 mm, concentrated in the months of October to April. Temperature oscillates between 29°C to 18°C, with higher temperatures coinciding with wetter months. The soil is a dark-red latosol very common in the Cerrado region. In this area only N_2 fixation by the soybean crop was investigated.

Biological nitrogen fixation by soybean was estimated using the ureide technique (Peoples et al, 1989) and by lupins using isotopic dilution of soil ^{15}N natural abundance (Shearer and Khol, 1986), using oats as the control. A N balance was performed for each crop considering the inputs (BNF contribution to the legumes and N fertiliser application to the cereals) and outputs of N in the grain harvest. It was assumed that there were no losses of the applied N fertiliser.

3. Results and Discussion

Nitrogen balance studies showed that soybean did not contribute to the build up of soil N reserves although the BNF contribution was between 150 and 300 kg $N.ha^{-1}$. The harvest index of N for soybean (76 to 84 %) was almost always greater than the percentage of N fixed in the plant (67 to

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81%), which resulted in negative balances to the system. Otherwise, soybean under zero-tillage consistently presented either a small positive, or a lower negative balance compared to the conventional tillage system (Figure 1). Although there was usually a negative N balance associated with the soybean, the subsequent wheat crop yielded 33% more grain than when it followed a fallow. Lupin was also used in the crop rotation, usually as a cover crop, so that all fixed N is carried over to the next crop (maize). In this study, lupin left nearly 250 kg N ha^{-1} in the residues, which resulted in a maize production of almost 10 ton ha^{-1} of grain (containing 150 kg N ha^{-1}), although only 80 kg N ha^{-1} of N fertiliser was applied.

These data show that the introduction of a legume cover crop will guarantee the maintenance of soil N reserves and the sustainability of crop production.

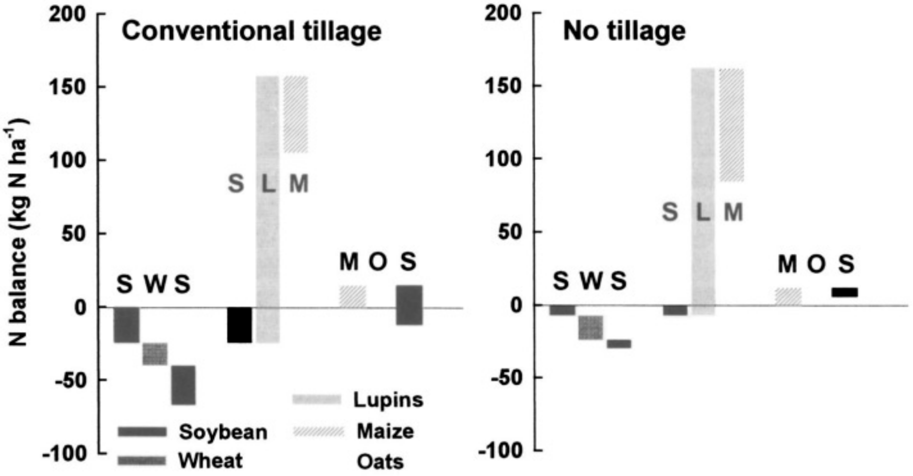

Figure 1. Nitrogen balance (kg N ha^{-1}) in three crop sequences (soybean-wheat-soybean; soybean-lupin-maize; maize-oat-soybean) established under conventional or no tillage planting systems in Londrina, PR (L. Zotarelli, ongoing MSc thesis, UFRRJ, Seropédica, Rio de Janeiro).

4. Conclusions

Under conventional tillage, although the contribution of BNF to soybean was high (190-315 kg N ha^{-1}), more N was removed in the grain, resulting in a negative balance. Under no-tillage, the N balance was occasionally slightly positive. In the case of lupins, as no N was harvested from the system, there was a large N contribution to the subsequent maize crop. The no-till system favoured the retention of soil N and a higher BNF contribution to the legumes.

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SELF REGULATION OF BIOLOGICAL N_2 FIXATION OF TREE LEGUMES IN A FOREST SUCCESSION OF THE EASTERN AMAZON.

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1. Introduction

As ever larger areas of primary forests are being converted for agricultural use it is increasingly important to understand the changes that occur in the nitrogen budget and in- and outputs of the ecosystem during the transition from primary forest via the field crop cycle to secondary forest.
We undertook extensive studies to quantify these changes at two study sites (Igarapé Açu and Peixe Boi) within the Brazilian state of Pará, in the micro-region *"Bragantina"*, one of the first regions of the Amazon to be colonized. Following a century of slash-and-burn practice, the area has lost most of its primary forest and may be representative of what large tracts of the Amazon will look like in the coming century. The established fallow system provides low but stable yields as long as fallow periods are maintained at 7 years or more. The slash-and-burn practice of land preparation leads to significant losses of nitrogen, particularly due to volatilization, estimated by Hölscher (1997) at 200 kg N ha^{-1} (for one 7-year-old secondary vegetation). Supposedly, these losses are recuperated by biological nitrogen fixation. Using the *natural abundance* ^{15}N *method* we assessed the biological nitrogen fixation capacity of native leguminous trees in the forest regrowth of the north-eastern Amazon to verify this supposition.

2. Material and Methods

In the context of this study, we took soil (0-10cm depth) and leaf samples randomly from three distinct phases of the *"terra firme"* secondary and primary forest, all situated on a nutrient-poor oxisol with a N content of around 0.11%. Secondary vegetation sites were grouped according to time since abandonment: 5-10 years (young), 15-25 years (intermediate), 35+ years (old). All samples were oven dried (60°C), then ground to a fine powder for analysis of carbon and nitrogen, and ^{15}N. Nitrogen isotope ratios ($^{15}N/^{14}N$) and total nitrogen contents were measured as described by Reineking et al. (1993), using an elementar analyser (Carlo Erba, NA1500) coupled to a mass spectrometer (Finningan, MAT 251). The $\delta^{15}N$ values of atmospheric N_2 ($\delta^{15}N = \pm 0$) and soil N are generally substantially different, and the distinct signals provide endpoints for nitrogen source identification.

3. Results

We found the $\delta^{15}N$ values of the young leaves from primary forest trees to be around 4.75‰ (n=45), irrespective of the plant family to which the species belonged. These values correspond with the range of $^{15}N/^{14}N$ ratio of their surface soils and suggest that in this climax vegetation all species derive their nitrogen from the soil. Contrary to this observation, sampled leaves of the young secondary forest show $\delta^{15}N$ values close to 0‰ (n=93) in Peixe Boi and approximately -1‰ (n=98) in Igarapé Açu (Table 1).
Because the isotopic composition of soil N vary from primary forest (6.74‰ ±0.16; n=30) to secondary forest, (3.57‰±0.11; n=60 in Igarapé Açu and 4.33‰±0.11; n=60 in Peixe Boi), we used the relationship between the N isotope composition of plants and soil, also called *"enrichment factor"* (ε_{p-s}) to trace the source of plant N (Mariotti et al. 1981). This can be approximated as the dif-

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ference between ^{15}N abundance in the substrate (i.e., total soil N) and the product (i.e., foliar N): $\varepsilon_{p\text{-}s} = \delta^{15}N_{leaf} - \delta^{15}N_{soil}$, when the substrate is a large reservoir with respect to the product. This factor is –2.0 in the primary forest vegetation, a difference attributed to ^{15}N fractionation in N mineralization and plant uptake. The higher plant-to-soil-difference for secondary forest (varying between 3.5 to 4.6) indicates a N-input into the system, probably with an origin in biological nitrogen fixation.

Table 1. N (%) and $\delta^{15}N$ (‰) of the topsoil (0-10cm) in relation to $\delta^{15}N$ (‰) of leaves from vegetation along chronosequences at Igarapé Açu and Peixe Boi. (Mean with standard error)

Study sites	Igarapé Açu			Peixe Boi			
	Secondary vegetation			Secondary vegetation			Primary forest
	young	intermediate	old	young	intermediate	old	
$\delta^{15}N_{soil}$	3.57^{a} (± 0.11)	3.28^{a} (± 0.11)	3.79^{b} (± 0.11)	4.33^{a} (± 0.11)	4.84^{b} (± 0.11)	4.26^{a} (± 0.16)	6.74^{c} (± 0.16)
$\delta^{15}N_{plants}$	-1.02^{a} (±0.14)	-0.20^{b} (±0.16)	0.25^{b} (±0.13)	0.11^{a} (±0.13)	1.00^{b} (±0.16)	0.60^{ab} (±0.19)	4.75^{c} (±0.14)
$\delta^{15}N_{plants} - \delta^{15}N_{soil}$	-4.6	-3.5	-3.5	-4.2	-3.8	-3.7	-2.0

4. Conclusion

There is little information in the literature to refute or support our findings. It is commonly accepted that tropical moist forests and the soil on which they grow accumulate large quantities of N during their growing stage, reaching steady state nutrient cycles upon maturation. At this point, we conclude from the data presented above, that symbiotic nitrogen fixation by leguminous trees is a process by which primary forests ensure rapid recovery from disturbances, natural or man-made, that cause serious losses of N from the system, e.g. burning, but most of these inputs and outputs are exceedingly difficult to quantify. However, the ecosystem appears to rely on a self-regulating mechanism whereby the rhizobial activity is inhibited by the accrual of N in the system and is rendered dormant, thus avoiding excessive N levels. Therefore, the determination of the $\delta^{15}N$ values of the plants and topsoil may provide a rapid appraisal of the role of symbiotic BNF in a forest stand. The enrichment factor may also serve as a quick tool to identify true primary forests.

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MULTIPURPOSE LEGUMES OF THE TROPICAL GENUS *CROTALARIA* ARE ASSOCIATED WITH UNUSUAL RHIZOBIA

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In tropical areas, spontaneous legumes play an important role in maintenance and improvement of soil fertility, but they remain largely unexploited. Among them, the legumes belonging to the genus *Crotalaria* are actively fixing nitrogen through the nodules they form on their roots in association with rhizobia. The genus *Crotalaria* is widespread in tropical regions and includes about 550 species in Africa and Madagascar (Polhill, 1982). These annual or perennial plants can be used in farming systems as green manure crops. Furthermore, they are also grown as multipurpose legumes because some of them can control the proliferation of important plant parasites such as nematodes. Up to now, *Crotalaria* were regarded as only nodulated by *Bradyrhizobium* strains belonging to the so-called cowpea miscellany group of rhizobia. We have recently shown that several species of *Crotalaria* exhibited a strong specificity for nodulation and did not nodulate with classical bradyrhizobia (Samba et al., 1999). In this paper, we report several examples of agronomic applications by *Crotalaria* used as fertilizers in the field. Biological nitrogen fixation (BNF) of three species of *Crotalaria* native to Senegal was measured using the N15 direct isotope dilution method. We also report the characterization of the specific *Crotalaria* nodulating-bacteria which form a new branch within the alpha proteo-bacteria distinct from other known rhizobia.

Numerous species of *Crotalaria* have been used as green manure all around the world. For example, in Sri-Lanka, *Crotalaria anagyroides* has been successfully used for soil improvement in tea plantations (Sandanam, 1976). In Brazil, *Crotalaria brevidens,* used as green manure in coffee plantations, provides an efficient soil cover, suppresses undesirable weeds, prevents soil erosion, and improves soil temperature and moisture which has a beneficial effect on the coffee crop (Muzilli, 1992). In Zambia, green manure *Crotalaria Zanzibarica* enhance the yield of beans (Mwambazi, 1998). Recently, in savanna regions of Ivory Coast, *Crotalaria juncea* has been planted to restore soil fertility in traditional upland rice-based cropping systems (Becker, 1999).

Another agronomic interest of *Crotalaria* is the property of some species to control nematodes populations. In 1983, Sano and Nakasano reported that the development of the larvae of *Meloidogyne incognita* inoculated to *Crotalaria spectabilis* was stopped at the3rd stage. In Brazil, experiments with *Crotalaria spectabilis* planted at 17 or 34 plants/m^2 strongly reduced population of *Meloidogyne incognita* (Huang, Tenante, 1981). In India, *Crotalaria Juncea* was recommended as safe crop for management of *Pratylenchus zeae* in sugarcane (Sundararraj, 1990). In USA, Florida, yield of eggplant and squash was reported to be enhanced after crop rotation with *Crotalaria Spectabilis* by depressing *Meloidogyne arenaria* populations (MacSorley, 1994).

However, caution is necessary when using Crotalaria because a few disadvantages can be encountered. In USA, Florida, *Crotalaria lanceolata* and C. *brevidens* became a new host plant for *Piezodorus guildini* an insect pest of soybean (Panizzi, 1985). In India, *Crotalaria juncea* can become a reservoir of entomophages, potential pests and plant diseases vectors (Sharma, 1997), and was shown to be a new alternative host of *Eurystylus oldi*, an important African pest of sorghum

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and castor plant (Maldes, 1998). A few Crotalaria, such as *Crotalaria retusa* can become an invading weed, and many species contain high level of alkaloids and are toxic for cattle, horses and other animals.

A greenhouse experiment was conducted to measure N2 fixation in three *Crotalaria* species, C. *perottetii,* C. *ochroleuca* and C. retusa, growing in Sénégal using direct isotope dilution technique. Two non-fixing plants, *Senna occidentalis* and *Senna obtusifolia* served as reference plants. Significant differences were observed between the three *Crotalaria* species. *C. ochroleuca* yielded more total nitrogen than did *C. perottetii* and *C. retusa.* The atom % 15N excess in the *Crotalaria* species was significantly lower than that of the reference plants, indicating that significant N2 fixation occurred in the three *Crotalaria* species. There was no significant difference in % Ndfa between the three Crotalaria species (47 % to 53 %). In contrast, genetic variability was observed based on total N fixed (Ndfa). *Crotalaria ochroleuca* significantly exhibited the higher amount of total N fixed, equivalent to 83 Kg of nitrogen per hectare.

117 rhizobial strains were isolated from 9 species of annual and perennial *Crotalaria* growing in Sénégal. According to their host range specificity, the strains and their *Crotalaria* hosts could be divided into two different groups:
-Group I includes fast-growing strains exclusively nodulating *C. perrottetii, C. podocarpa* and *C. glaucoides* These fast growing rhizobia were highly specific.
-Group II includes slow-growing strains nodulating *C. comosa, C. goreensis, C. hyssopifolia, C. lathyroides, C. ochroleuca, C. retusa* and also typical cowpea type host plants such as *Macroptilium atropurpureum* and *Acacia albida.* These slow-growing strains were promiscuous and resembled typical bradyrhizobia.
Taxonomical position of all isolates was studied by SDS-PAGE. Results showed 2 main electrophoretic groups at a level of similarity of 90%. Slow-growing *Crotalaria* rhizobia formed an electrophoretic group with the wild type strain of *B. japonicum* (LMG 6138). All fast-growing *Crotalaria* strains were included in an homogenous electrophoretic group well separated from other known species of rhizobia. Analysis of 16 S rDNA sequences of two representative strains of each group confirmed that the slow-growing strains belonged to *Bradyrhizobium Japonicum,* and surprisingly that the specific fast-growing strains belonged to the *Methylobacterium* genus, thus constituting a new branch of nodulating bacteria.

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NITROGEN FIXATION BY FORAGE LEGUMES AS A DRIVING FORCE BEHIND THE RECUPERATION AND IMPROVEMENT OF SOIL QUALITY IN TROPICAL AGRICULTURAL SYSTEMS: OPPORTUNITIES FOR WIDER USE OF FORAGE LEGUMES?

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As we move towards the next millennium the demand for livestock products is outstripping that of any other agricultural product. Growth rates for meat and milk averaged 3.9% for 1990-95 in developing countries where most of the expected increases in population, income and urbanization will occur. About 60% of the world's agricultural land is used for grazing. Much of this area is used for extensive low-input systems on marginal lands. These areas are undergoing varying degrees of land degradation as livestock are forced onto more fragile lands to make way for cropping on the better lands. These trends are causing the concern that increased livestock production will be associated with further environmental degradation (FAO, 1999).

The presence of 20 to 40% of forage legume in tropical pastures has been shown to dramatically increase animal meat and milk production by as much as ten-fold (Thomas et al., 1995). The potential inputs of nitrogen via legume biological fixation in such pastures can balance the N cycle (Thomas, 1995). More recent evidence indicates that forage legumes can also enhance the remedial role of forage grasses in terms of soil quality. Work from long-term experiments on pastures and crop-pasture rotations conducted in the Colombian llanos and Brazilian cerrados by teams of scientists from national programs, international agricultural research centres and specialized research organizations has documented the benefits of pastures containing nitrogen-fixing forage legumes (Table 1 and Thomas, Ayarza, 1999).

In spite of these demonstrated benefits the adoption of forage legumes in grazing systems by farmers is low. For example, although there was a 10-fold increase in the adoption of improved grass pastures over a 16-year period in the Colombian llanos, only 2% of the pasture area contained legumes and 88% of farmers reported that legumes failed to persist (Smith et al., 1997). Other constraints to the adoption of forage legumes such as a lack of seed supplies, poor acceptability by farmers, have been discussed by Thomas (1995).

To improve this situation increased awareness of the multiple beneficial effects of forage legumes for productivity and for soil improvement is required at farmer and policy maker level. Recent concern over the environmental effects of livestock producion systems has highlighted other potential beneficial effects of improved pastures in terms of greenhouse gas emissions and land degradation (FAO, 1999). Unless more attention is focused on these issues it is unlikely that the advances in knowledge of biological nitrogen fixation evident in this congress, will result in the increased use of forage legumes in grazing systems. Rather efforts should be made to achieve better adoption and use of forage legumes by means other than the promotion of nitrogen fixation *per se.*

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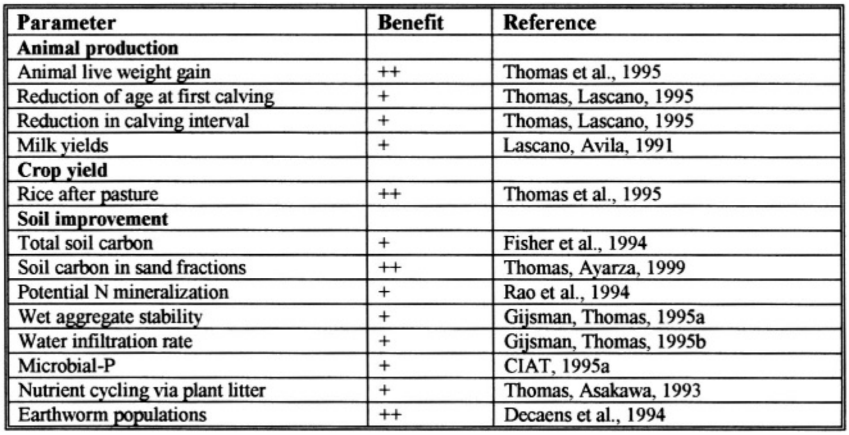
Conclusion and priorities

Forage legumes can improve productivity and soil quality when introduced into tropical pastures. However there is a need to focus further efforts as a result of poor adoption by farmers and a marked reduction in the efforts of researchers worldwide on this topic.

Extensive grazing systems only provide around 9% of the world's beef whereas mixed farming systems produce 54% of the meat and over 90% of the milk (FAO, 1999). Farmers practicing mixed farming are in regions where land expansion is no longer possible and intensification is one of the few options available to meet demands. It is in these systems where the opportunity to introduce forage legumes is greatest (Smith et al., 1997). Efforts to increase knowledge and awareness of the multiple beneficial effects of forage legumes (and not just their nitrogen-fixing abilities) should be targeted primarily to these mixed farming systems in order to reap the benefits of biological nitrogen fixation. Specific opportunities include ground covers in plantations, trees/shrubs for "cut and carry" systems, crop-pasture rotations, minimum and no-tillage systems, recuperation of degraded lands, agroforestry and multi-strata home gardens.

Table 1. Beneficial effects of grass/legume pastures on productivity and soil quality of agropastoral systems (* see Vera, 1998 for references in Table)



Parameter	Benefit	Reference
Animal production		
Animal live weight gain	++	Thomas et al., 1995
Reduction of age at first calving	+	Thomas, Lascano, 1995
Reduction in calving interval	+	Thomas, Lascano, 1995
Milk yields	+	Lascano, Avila, 1991
Crop yield		
Rice after pasture	++	Thomas et al., 1995
Soil improvement		
Total soil carbon	+	Fisher et al., 1994
Soil carbon in sand fractions	++	Thomas, Ayarza, 1999
Potential N mineralization	+	Rao et al., 1994
Wet aggregate stability	+	Gijsman, Thomas, 1995a
Water infiltration rate	+	Gijsman, Thomas, 1995b
Microbial-P	+	CIAT, 1995a
Nutrient cycling via plant litter	+	Thomas, Asakawa, 1993
Earthworm populations	++	Decaens et al., 1994

+ = an increase of <100% compared with grass-only or native savanna pastures

++ = an increase of >100% compared with grass-only or native savanna pastures

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SOIL FERTILITY IMPROVEMENT AND ECONOMIC RETURNS FROM INCORPORATING LEGUMES IN CROPPING SYSTEMS IN THE SEMI-ARID TROPICS

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Despite the considerable progress made in breeding for high-yielding, high nitrogen-fixing, pest and disease resistant leguminous crops, yields realized by the small holder farmers in the semi arid tropics remain in the range of 20-39% of those achieved on experimental research stations. Low soil nutrient and moisture availability, and socio-economic constraints to adoption by farmers are some of the major causes for this yield gap. Available data shows that where traditional farming practices prevailed, the land area sown to legumes is far smaller than that sown to non-legumes. Farmers are not keen to include legumes in their cropping systems probably because:

Farmers require more cereals for households consumption than legumes. In areas where double cropping is not feasible, many resource-poor farmers cannot allocate much land for legumes so that they have to wait the next year to reap a good harvest of cereals. A probable solution is cereal/legume intercropping instead of cereal/legume rotation.

Production of legumes is relatively more risky than of cereals (sorghum and maize). The price and yield risk of legumes are much higher than those of cereals. Some of the constraints and socio-economic aspects of this risk have been discussed in detail by Joshi and Pande (1996).

Fewer efforts to enhance legumes than cereal yields. A major breakthrough has been achieved in enhancing yields in such cereals as maize and wheat, but not such breakthrough has occurred for legumes. Short-duration legumes bred for irrigated agriculture are usually not suitable as intercrops in rained areas.

Inadequate price support mechanisms to alleviate effects of market price fluctuations in legumes. Price stability for legumes is a policy matter that should be addressed by governments of countries in the semi arid tropical regions.

Scarce economic analysis of direct and indirect benefits of legume in cropping systems. The residual benefits of legume need to be costed. Analytical and quantitative data from farmers' fields have shown the beneficial effects of legume in sustaining the fertility status of soils, saving inorganic N fertilizer in subsequent non-legume crops. The beneficial monetary returns gained by farmers incorporating grain legumes in the cropping systems are relatively high in semi-arid tropical environments. These results need to be made known to a wide audience of policymakers and farmers.

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DIVERSITY OF INDIGENOUS FIELD ISOLATES OF COWPEA BRADYRHIZOBIA FROM ECOLOGICAL ZONES OF GHANA

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Most African soils harbour a large number of rhizobia that fix nitrogen in symbiosis with agriculturally important food legumes. However, very little is known about the extent of diversity of the indigenous rhizobia in tropical soils (Mpepereki et al., 1997). Our aim has been to study the phenotypic and genotypic variations of indigenous bradyrhizobia nodulating cowpea, *Vigna unguiculata,* in two different ecological zones of Ghana.

Analysis of the diversity of cowpea rhizobial strains from different sites in the Transitional and Northern Savanna Zones of Ghana is done by rep-PCR using ERIC primers (de Bruijn, 1992), intrinsic antibiotic resistance, growth characteristics on YEM plates containing Congo red and symbiotic performance in magenta jars under controlled conditions. ERIC-PCR analysis of 58 rhizobial isolates showed high diversity among isolates, with a large number of fingerprint patterns (Fig. 1). Growth on YEM plates revealed both fast- (68%) and slow-growing (32%) isolates. Fast-growing isolates formed large, slimy colonies while slow-growing types produced mostly small, dry colony types. Generally, there were significant variations in the total dry matter and nodule number produced by both fast- and slow-growing rhizobial inoculants. Fast-growing rhizobial isolates were generally more sensitive to the antibiotics, tetracycline > streptomycin > nalidixic acid, compared to slow-growing types; though both showed less sensitivity to ampicillin and chloramphenicol.

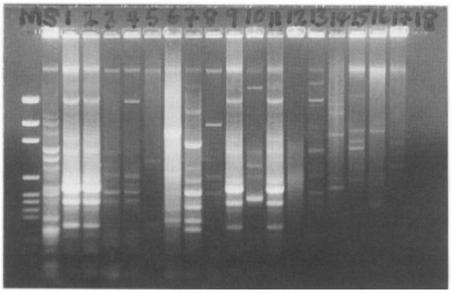

Fig. 1. ERIC-PCR fingerprint patterns derived from pure cell cultures of 18 selected cowpea rhizobial isolates. Lane M= molecular weight marker; laneS= TAL169 and lane 1= LBG13 are reference strains; lanes 2-17= indigenous field isolates and lane 18= negative control with no template DNA.

On-going and future work will involve: further rep-PCR analyses of cowpea isolates; diversity studies of cowpea isolates collected from the Northern Savanna Zone of Ghana; determination of rhizobial numbers, pH, nitrogen and phosphorous content of soils from sites in the Transitional and Northern Savanna Zones; competitiveness and symbiotic performance of isolates in field conditions.

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THE INFLUENCE OF SOIL *RHIZOBIUM* POPULATIONS ON THE NODULATION OF ALTERNATIVE CLOVERS IN SOUTHERN AUSTRALIA

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1. Introduction

Alternative clovers are currently being evaluated in southern Australia for their potential to increase legume diversity in pastures. Recently, a survey indicated that 25 % of sites in a target growing area with annual rainfall of 300-400 mm and strongly alkaline soils, had large indigenous *Rhizobium* populations ($>10^3$ / g soil) that were poor in N_2 fixation (Denton et al., in press). The aim of the study reported here was to determine what influence these indigenous populations had on the nodule occupancy of clovers inoculated with commercial *R. leguminosarum* bv. *trifolii.*

2. Materials and Methods

The ability of indigenous *Rhizobium* to compete for nodulation with introduced inoculant strains under alkaline soil conditions was determined by inoculating 3 clover species with 2 commercial rhizobia (Table 1) in a field trial. Nodule occupancy was determined using PCR with the *nifH*-directed primer, RP01 (Richardson et al. 1995) to identify nodule isolates and results were verified using REP and ERIC primers (de Bruijn, 1992).

3. Results and Discussion

Strain TA1 could not be recovered from nodules of *T. purpureum* or *T. resupinatum* in the year that plants were inoculated, though this strain did successfully nodulate T. *alexandrinum* (Table 1) However, TA1, and also WU95 had poor persistence into the second year, on nodulating regenerating clovers. In contrast, CC2483g and WSM409 were persistent in the second year (Table 1). A dominant field isolate distinct from commercial strains, nodulated all clover species and occupied 27 % and 36 % of nodules from uninoculated plots in year 1 and 2, respectively.

Table 1. Nodule occupancies of 3 clover species each inoculated with 2 commercial *Rhizobium*.

Clover Species	Inoculant *Rhizobium* Strain	Inoculant recovery 1997	Inoculant recovery 1998
T. alexandrinum	TA1	39 %	3 %
T. alexandrinum	WU95	36 %	0 %
T. purpureum	TA1	0 %	0 %
T. purpureum	WSM409	31 %	30 %
T. resupinatum	TA1	0 %	0 %
T. resupinatum	CC2483g	21 %	27 %

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STUDIES ON NITROGEN FIXATION IN AMAZONIAN FLOODPLAIN FORESTS

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1. Introduction

Amazonian floodplains cover an area of about 300 000 km². Parts of the floodplains are the várzea, which is rich in nutrients and suspended matter, and the igapó, which is poor in both. Floodplain forests are dynamic and high productive ecosystems, closely linked with the supply of nitrogen.

2. Material and Method

Leaves were collected in a várzea forest on Ilha de Marchantaria, at Tarumã Mirím (igapó) and Ariaú, an igapó area which is influenced by white-water through small creeks. Plant material was dried at 60 °C, finely ground and analysed using an elemental analyser in combination with a mass spectrometer. The results are expressed in $\delta^{15}N$ with low values indicating N_2 fixation (Shearer, Kohl, 1988).

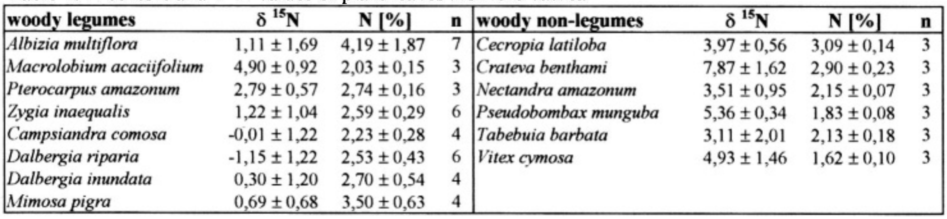
3. Results and Discussion

Low $\delta^{15}N$ values were obtained from legumes as well as from non-legumes, indicating that not only legumes profit from N_2 fixation. From October '98 until April '99 no seasonal variation in N_2 fixation was apparent and no significant differences in $\delta^{15}N$ values between várzea and igapó plants were measured but total nitrogen content is considerably lower in plants from the igapó. Among the woody legumes studied low $\delta^{15}N$ values could be measured in trees, bushes and lianas (Table 1).

The $\delta^{15}N$ in *Macrolobium* was relatively high and did not differ from non-legume $\delta^{15}N$, which is in accordance with nodulation observations. The group of non-leguminous trees has mean $\delta^{15}N$ values between 3,1 and 7,9 in leaves. Exceptions in this group with relatively high $\delta^{15}N$ values are flooded *Cecropia* trees in igapó and *Tabebuia barbata,* which have low $\delta^{15}N$ values similar to those of N_2 fixing legumes. This phenomenon has been described earlier for flooded *Cecropia* trees in the várzea (Kern et al.; in press). One explanation might be the activity of associated N_2 fixing bacteria in the root systems. However this is not clarified yet and needs further research.

It is concluded that with about 20 % legume trees N_2 fixation may play an important role as a nitrogen source for Amazonian floodplain forests. There are indications of N_2 fixation in legumes and in non-legumes. Intraspecific and interspecific differences in N_2 fixation seem to be more important than local or seasonal variabilities.

Table 1: N content and $\delta^{15}N$ values of plant leaves from the várzea



woody legumes	$\delta\ ^{15}N$	N [%]	n	woody non-legumes	$\delta\ ^{15}N$	N [%]	n
Albizia multiflora	1,11 ± 1,69	4,19 ± 1,87	7	*Cecropia latiloba*	3,97 ± 0,56	3,09 ± 0,14	3
Macrolobium acaciifolium	4,90 ± 0,92	2,03 ± 0,15	3	*Crateva benthami*	7,87 ± 1,62	2,90 ± 0,23	3
Pterocarpus amazonum	2,79 ± 0,57	2,74 ± 0,16	3	*Nectandra amazonum*	3,51 ± 0,95	2,15 ± 0,07	3
Zygia inaequalis	1,22 ± 1,04	2,59 ± 0,29	6	*Pseudobombax munguba*	5,36 ± 0,34	1,83 ± 0,08	3
Campsiandra comosa	-0,01 ± 1,22	2,23 ± 0,28	4	*Tabebuia barbata*	3,11 ± 2,01	2,13 ± 0,18	3
Dalbergia riparia	-1,15 ± 1,22	2,53 ± 0,43	6	*Vitex cymosa*	4,93 ± 1,46	1,62 ± 0,10	3
Dalbergia inundata	0,30 ± 1,20	2,70 ± 0,54	4				
Mimosa pigra	0,69 ± 0,68	3,50 ± 0,63	4				

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BIOLOGICAL NITROGEN FIXATION BY *CROTALARIA JUNCEA* AND *MUCUNA NIVEUM* DETERMINED USING THE ^{15}N NATURAL ABUNDANCE TECHNIQUE

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Crotalaria juncea and *Mucuna niveum,* have been widely used as green manures because of their high dry matter production and ability to fix nitrogen biologically. To quantify the nitrogen fixation during the growth cycle of these legumes, the ^{15}N natural abundance technique was employed using five reference plants. It is known that different species could vary in their δ^{15}N values (Sandford, et al, 1994) depending on the isotopic fractionation occurring during the N uptake and assimilation (Kohl and Shearer, 1980). So it is recommended that many different non-N_2-fixing references plant should be collected to determine the magnitude of this variation (Shearer and Kohl, 1988). Peoples et al, (1989) mentioned that enough differences between ^{15}N natural abundance of N derived from soil and atmosphere is necessary to get good precision on the application of the technique.

A field experiment was carried out on a sandy soil series Ecologia (Abruptic Arenic Ochraquult). Legumes were inoculated with appropriate *Bradyrhizobium* strains and cultivated in plots of 8 x 10 m in rows spaced 0,5 m, with four replicates. Above ground parts were collected during the legume growth from 15 to 60 days after planting (DAP) at different intervals for *C. juncea* and *M. niveum.* Reference plants: *Zea mays, Sorghum vulgare, Cynodon dactylon, Cyperus rotundus* and *Sida glaziovii* were collected at the same dates. All materials were dried, weighed and finely ground to analyze total N and δ^{15}N. The fractionation coefficient B was assumed to be –1.00%.

δ^{15}N values of reference plants showed small variation between different collection times into the same specie except for *C. rotundus* that showed the high standard error of the mean (6,246 ± 1,72). Among species, *Z. mays* showed the highest mean δ^{15}N value (8,054 ± 0,49) while *S. vulgare* presented the lowest (5,204 ± 0,304) providing the maximum and minimun estimates of FBN in the legumes, respectively. The other reference plants gave intermediate estimates. As the proportion of BNF was greater the difference was minimized because of the maior range between isotopic composition of non-fixing and fixing plants. The proportion of N_2 fixed by *C. juncea* ranged from 38 to 58 % at 15 DAP estimated with *S. vulgare* and *Z. mays,* respectively. At 49 DAP *C. juncea* showed the high proportion of BNF estimated as 84 and 90 %. *M. niveum* showed proportions of N fixation at 15 DAP in a range of 64 to 76 % with *S. vulgare* and *Z. mays* as reference plants, respectively, and at 42 DAP in a range of 90 to 94%. The amount of fixed N by *C. juncea* and *M. niveum* reached 98 and 56 kg ha^{-1} at 60 DAP, respectively. The ^{15}N natural abundance technique was found to be appropriate for estimating the proportion of N_2 fixed by the legumes. The non-fixing reference plants used were adequate since no significant differences were detected between the five independent estimates of BNF by *C. juncea* and *M. niveum.* Both legumes obtained large proportions of their N from BNF suggesting that they are potencially valuable as green manures.

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DIVERSITY AND ABUNDANCE OF POPULATIONS OF BEAN-NODULATING RHIZOBIA AS A FUNCTION OF LIMING AND CROPPING HISTORY IN ACIDIC BRAZILIAN SOILS

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The application of lime to acidic soils may provide nutrients and favourable conditions to kick-start the growth of bacterial cells in the short term, while long term alteration due to liming may also have an impact on diversity of naturalised symbiotic rhizobial populations. The aims of this study were: (i) to determine the effects of cropping and liming on the the abundance and survival of bean-nodulating rhizobia in an acidic soil; (ii) to determine the structure of bean-nodulating populations along gradients of increasing acidity stress in a Brazilian soil subjected to liming and cropped with common bean (*Phaseolus vulgaris* L.). Rhizobial populations were smallest in soils continuously cropped with rice compared with those cropped with common bean, maize and a soybean/wheat rotation. In the absence of a common bean crop the effects of the soil acidity complex on declines in the size of rhizobia populations were highly significant After incubation rhizobia in an acidic soil sampled from the bean field remained at around 90 to 94% of the initial population in limed soils, (4, 7 and 11% of aluminium saturation), whilst in soils with 27 and 36% of aluminium saturation the abundance declined to about 3 to 7% of that initially observed. Polymerase chain reaction (PCR)-mediated restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer (IGS) and the 16S rRNA gene indicated that the structure and diversity of the native rhizobia from the bean field was altered due to liming. The population in the unlimed soil had less diversity than in limed soil. Isolates belonging to *R. tropici* IIB and to *R. leguminosarum* bv. *phaseoli* putative species were predominant (49.7% and 32.6%, respectively). The number of isolates/IGS groups within *R. leguminosarum* bv. *phaseoli* profiles gave a negative relationship with the number of rhizobia in the soil; whilst with *R. tropici* IIB the correlation between number of IGS groups within each population and density of rhizobia populations was positive. The expected dominance of *R. tropici* in the rhizobial population from unlimed soil, hypothesised on the basis of earlier reports of acid tolerance in this species, was not found. Isolates affiliated to *R. tropici* IIB and to *R. leguminosarum* bv. *phaseoli* were predominant in all soil populations (50% and 33%, respectively overall). In the soil which had received liming the *R. tropici* IIB pattern types contained the highest diversity in terms of the number of IGS groups. In contrast, there was a higher number of *R. leguminosarum* bv. *phaseoli* types in the unlimed soil with lowest pH than in the populations from soils with the highest pH. Effects of decreased acidity on increased abundance of rhizobia and diversity of rhizobial populations were found, but there was no clear relationship between tolerance of the rhizobial isolates of acidity in laboratory tests and the acidity of the soil from which they were isolated. The high diversity presented by these indigenous rhizobial populations is unlikely to enhance nitrogen and consequently to improve bean production directly; but this study is the first step in building knowledge that will help us to understand what makes the rhizobia-legume symbiosis with indigenous or selected inoculated strains succeed or fail in the field. The genetic variability demonstrated by bean-nodulating rhizobial populations for small-scale agricultural systems in the tropics could be a useful buffering against soil stresses.

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LEGUME INOCULATION AND OVERCOMING CONSTRAINTS TO INOCULANT USE: ACTIVITIES OF THE RHIZOBIUM ECOLOGY NETWORK OF EAST AND SOUTHERN AFRICA

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The Rhizobium Ecology Network of East and Southern Africa (RENEASA) seeks to increase biological nitrogen fixation by legumes in agroecosystems by identifying and better understanding the conditions that underlie successful use of legume inoculants. Legume inoculant are being, or have been, produced for distribution to farmers in Kenya, Malawi, Rwanda, South Africa, Tanzania, Zambia and Zimbabwe. This production in East and Southern Africa stands in contrast to that in West and Central Africa where inoculant technologies are regarded as unnecessary or "inappropriate", This in part is because of the differences in the origins and symbiotic requirements of cultivated legumes. The highlands and plateaus of East and Southern Africa provide habitat for several exotic legumes with specialized rhizobial requirements including bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), soyabean (*Glycine max*) and the pasture legumes (*Medicago sativa* and *Melilotis* spp.). Legume inoculant industries have developed around the specialized rhizobial requirements of commonly grown legumes in East and Southern Africa. Woomer et al. (1998) estimated that 160 tonnes of inoculants per year were being produced in the sub-region. Opportunities for the production of legume inoculants have attracted scientists.and lead to the increase of expertise in rnizobiology. Inoculant technology is therefore available throughout the sub-region.

Soils of known rhizobial population sizes (Woomer *et al.,* 1997) were recovered from sites and transported to the glasshouse. Three treatments were imposed: a complete control (C), + rhizobial inoculant (+R) and urea application (+N). Six hosts were studied, two indigenous species (*Vigna unguiculata and Arachis hypogaea*) and four exotic to Africa (*Glycine max, Phaseolus vulgaris, Pisum sativum* and *Leucaena leucocephala).* Pots were arranged in a randomized, complete block design with four replicates. After thirty-five days, plants were harvested and nodules recovered. Cross-site comparisons were made by expressing results on a relative basis (e.g. inoculation response = (R-C)/C).

Legumes responded to inoculation with 32-125% increase in growth and 0-78% increase in the frequency of effective nodules compared to the uninoculated control. *Glycine max* was the most responsive host (125%). The ratio of inoculation response to urea response is an indirect measure of symbiotic potential, a 1:1 ratio suggests that hosts are equally adept at N-fixation and urea N-assimilation. Values obtained were within 10% of unity for 3 of 6 host legumes. The low value for *P. sativum* (0.59) suggests that either the inoculant is not fully effective or the host uses N-fertilizer efficiently. The relatively high values for *P. vulgaris* and *L. leucocephala* may be attributed to the poor suitability of urea as an N source.

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THE EFFECTS OF CHLORSULFURON ON THE ABILITY OF *RHIZOBIUM* TO INFECT CHICKPEA (*CICER ARIETINUM*) ROOTS

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1. Introduction

Chlorsulfuron is an acetolactate-synthase inhibiting (ALS) herbicide used for controlling broadleaf weeds in cereal crops. This herbicide has been found to persist in alkaline soils of southern Australia and affect the growth of subsequent legume crops and pastures in the rotation. Chlorsulfuron has been found to inhibit nitrogenase activity of alfalfa (Martensson, Nilsson, 1987). However, the mechanism responsible for this inhibition is unclear. The reduction may be due to direct ALS inhibition in the plant, possible toxic effects on the *Rhizobium* or an interference on the symbiotic nitrogen fixation process.

To investigate the effects of Chlorsulfuron on the ability of *Rhizobium* to effectively nodulate chickpea plant roots.

2. Methods

Rhizobium were cultured in the presence or absence of Chlorsulfuron (0.01 ug ai ml^{-1} equivalent to recommended application rate) for two days in yeast mannitol broth. Prior to inoculation, the *Rhizobium* cultures were rinsed three times with ¼ Ringer's solution and then inoculated into the pots. Seeds were pre-germinated for three days on agar plates in the presence or absence of chlorsulfuron (0.01 ug ai ml^{-1} of agar equivalent to 10% recommended application rate) and were planted into pots in which chlorsulfuron was present (0.001 ug ai g^{-1} of soil; equivalent to 10% recommended application rate) or absent. Uninoculated and nitrogen controls were included. All treatments were replicated five times. The experiment was carried out in a randomised block design in a controlled environment room (25°C max and 16°C min with 12 hour day length).

3. Results

Chlorsulfuron significantly decreased the number of nodules formed on chickpea roots regardless of the stage of growth to which it was applied (four way interaction effect between nitrogen and herbicide presence in *Rhizobium,* germination and pot p= 0.05). This included the pre-exposure of *Rhizobium* to the herbicide. Chlorsulfuron also significantly reduced the biomass of both shoots and roots of chickpea plants.

4. Discussion

The results indicate that chlorsulfuron is inhibiting both growth and nodulation of chickpea plants. When chlorsulfuron was present only during the culture of *Rhizobium* (ie herbicide not present at any other stage) the number of nodules was significantly reduced when compared to the control plants. This suggests that chlorsulfuron was affecting the ability of the *Rhizobium* to effectively infect chickpea plant roots.

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Acknowledgments

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BRAZILIAN TRIALS TO EVALUATE THE EFFECTS OF SOYBEAN REINOCULATION

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1. Introduction

In Brazil, large scale commercial crop production started in the early 1960s. Soils were originally free from soybean bradyrhizobia, therefore, during the establishment of the crop, the seeds had to be inoculated. The inoculants were brought especially from the USA, but a search for strains adapted to the Brazilian conditions started immediately. The inoculants sold in Brazil must obligatorily carry two of the four strains recommended by a National Committee of Rhizobiologists. Soybean inoculation is required when the crop is introduced in a new area, but today most of the 13 million hectares cultivated with this legume were previously inoculated and show a high population of naturalized bradyrhizobia (10^3 to 10^6 cells g^{-1} of soil). It was then necessary to evaluate if soybean reinoculation in these areas would bring benefits to the plant N nutrition.

2. Material and Methods

A National net trial was established and from 1993 to 1996 thirteen experiments were performed in seven counties located in the main producing areas. The strains evaluated are those recommended for the use in commercial inoculants in Brazil: *Bradyrhizobium elkanii* SEMIA 587, SEMIA 5019 (=29w) and SEMIA 5079 (=CPAC 15) and *B. japonicum* SEMIA 5080 (=CPAC 7). All combinations of the four strains were tested. Peat based inoculants (10^8 to 10^9 cells g^{-1}) of single strains were prepared annually by FEPAGRO (Porto Alegre, RS). The inoculants were distributed to all partners one month before planting. At planting, equal proportions of inoculants were mixed. The inoculation procedure consisted of adding 500 g of peat inoculant:500 ml of a sugar solution (10% to 25% in water): 50 kg of seeds. Non-inoculated control treatments were also included, receiving or not N fertilizer. In each state, a recommended cultivar for that area was used.

3. Results

In nine of the 13 experiments, the reinoculation significantly increased yield and total N content of grains (Duncan, $p<0.05$). In most cases, reinoculation also increased nodule number and nodule mass, as well as nodule occupancy by the inoculated strain. Considering the 13 experiments, reinoculation increased yield up to 23% and total N in the seeds up to 25%. The best strain combination, SEMIA 587 + SEMIA 5080, increased yield by an average of 7.8% and 3.8% in the South and Central Regions of Brazil, respectively. Often, a positive residual effect of nitrogen fixation was observed on the crop that followed soybean, wheat. Addition of a small amount of N fertilizer at sowing (20 to 40 kg of N ha^{-1}) to avoid a possible initial stage of N starvation, did not result in any increase in yield. The addition of high levels of N fertilizers (200 to 400 kg of N ha^{-1}) decreased drastically nodulation and also did not result in any increase in yield. The release of these results stimulated the practice of soybean reinoculation and the number of farmers using this technology increased from 28 to 60%.

4. Conclusions

Although Brazilian soils are very poor on N, the nitrogen fixation process is able to fully supply the N plant demands. Reinoculation with selected strains promotes increases in nodulation, nodule occupancy and yield.

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NITROGEN FIXATION AND N BALANCE IN SUBTERRANEAN CLOVER PASTURE ASSOCIATED WITH ANNUAL AND PERENNIAL GRASSES

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Annual grasses have traditionally coexisted with subterranean clover in the permanent pastures of southeastern Australia. However, these pastures have proven unsustainable due to accelerated soil acidification, which has reduced productivity and pasture quality. Although clover is important for economic production of pasture, it has increased soil inputs of N, enhancing nitrification and the amount NO_3^- leached. The later process has caused soil acidification (Ridley *et al.* 1999). Perennial grasses are being increasingly substituted for annual grasses to help reduce NO_3^- leaching. Lime application is recommended to ameliorate the acid soils. The purpose of this study was to investigate these management changes on clover N fixation and the net total N balance.

Field experiments were conducted near Wagga Wagga in southern NSW between April 1994 to October 1996. There were four treatments: annual pasture without lime (AP–), annual pasture with lime (AP+), perennial pasture without lime (PP–) and perennial pasture with lime (PP+). The lime treatment was applied prior to pasture establishment, to achieve a pH of 5.5 in the top 10 cm. The aboveground N_2 fixation of subterranean clover was estimated by the ^{15}N natural abundance technique. The net N balance was estimated by mass balance.

Table 1. Fixed N and net total N balance in an annual and permanent pasture. Values are totals (kg N ha^{-1}) for the period of 1994.4-1996.10.

	AP–	AP+	PP–	PP+
N_2 Fixation[1]	220	293	189	230
Wet deposit[2]	14	14	14	14
Gaseous N loss	7	19	25	37
Nitrate leaching[3]	48	67	31	41
Product removal	20	24	21	24
Net balance	159	199	126	148

[1]Corrected for 30% below-ground fixed N contribution; [2] based 5.6 kg N/y for 2.5 years; [3]through deep drainage and lateral flow (White, 1999).

The proportion of clover N derived from N_2 fixation reached peak levels of 0.85-0.95 during spring, with little difference between treatments (data not presented). Less N was fixed by clover associated with perennial grasses, 189-236 kg N ha^{-1}, than with annual grasses, 220-295 kg ha^{-1}, and less in unlimed than limed pastures (Table 1). The gaseous N losses (mainly by NH_3 volatilisation) were higher in perennial than in annual pastures, and higher in limed than in unlimed pastures. Nitrate losses were also higher in limed than in unlimed pastures, but lower in perennial than in annual pastures. N balances were positive for all pastures, with the same order as the N_2 fixation among the four pastures. Less net N built up in soil and lower NO_3^- losses (c.37%) under perennial pastures will result in lower rates of soil acidification compared to annual pastures. With substantial positive net total N balances in all pastures, a shift in pasture composition to greater non-legume dominance is anticipated.

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EFFECTS OF SOIL TILLAGE MANAGEMENT AND CROP ROTATION ON BRADYRHIZOBIA POPULATION

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1. Introduction

Different tillage and crop rotation systems affect soil sustainability. Conventional tillage (CT) with traditional plowing and disking, reduces soil organic matter and increases erosion, especially in the tropics. As an alternative, the no-tillage (NT) management, sowing directly through the mulch, protects the soil against erosion by water, improves soil structure stability and moisture content and, with time, also increases soil organic matter contents and microbial biomass. The NT system is used today in 30% of the cropped area in Brazil. Several combinations of legumes and non-legumes are also used in multiple cropping systems, contributing to the regenerative processes that must operate in a sustainable agricultural system. However, the level of bradyrhizobia diversity and nitrogen fixation rates under those agricultural management systems has yet to be determined and some studies conducted in Brazil are described here.

2. Material and Methods

The study was performed in an area which was cultivated with soybeans for more than 30 years, had not received any inoculant for the past 15 years and was under NT and CT and the crop rotations [soybean (*Glycine max*)/wheat (*Triticum aestivum*)/maize (*Zea mays*), S/W/M; soybean/wheat, S/W; maize/wheat, M/W] for the last 17 years. Five soil samples (each with 10 samples randomly collected within each plot) were recovered from each of three replicates at the depth of 0 to 15 cm, and mixed on a sterile bag. The number of bradyrhizobia cells was evaluated by the MPN counting technique and, from each treatment, 25 nodules were randomly collected from the dilution 10^{-2}, totaling 150 nodules. The strains were isolated from these nodules.

3. Results

The number of soybean bradyrhizobia cells was higher under the NT and under the crop rotations including soybean and 142 bradyrhizobia strains isolated from the nodules were characterized. Of the four methodologies employed, morphological characterization, serological reaction, protein profile, Nod factor profile and DNA fingerprinting after PCR reaction with arbitrary short primers (RAPD), the last one was the most discriminating to characterize the isolates. Using the RAPD, the 142 isolates have shown 16 distinct genomic patterns. Those 16 DNA genotypes have also been grouped into five protein and three Nod factors profiles. A higher diversity was observed in soils under NT with the crop rotations with S/W/M, while the lowest diversity occurred in soils under CT and the M/W rotation. The presence of soybean in the crop rotation system significantly affected the bradyrhizobia diversity, increasing the number of genomic patterns. A greenhouse experiment with the isolates has shown that some of them were as effective as the four strains which are currently in use in Brazilian commercial inoculants The most efficient isolates were found in the NT and crop rotation systems including soybean.

4. Conclusions

The tillage and crop rotation management systems affected quantitatively and qualitatively the soybean bradyrhizobia population. More benefits to the bradyrhizobia population were verified in the no-tillage with crop rotations including soybean at least every other year.

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INTERACTIONS AMONG RHIZOBIA AND BIOCONTROL AGENTS IN FORAGE LEGUMES

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Forage legumes play an important role in the agriculture of Uruguay as a major source of high quality feed for livestock. Their nitrogen fixation capability in symbiotic association with rhizobia confers them a beneficial carryover effect in crop rotation. Nevertheless, important losses in pasture production are due to soilborne fungal pathogens (Altier 1996). The utilization of rhizospheric fluorescent pseudomonads as biocontrol agents is a feasible alternative to solve this problem.

Three native *Pseudomonas fluorescens* strains (UP61, UP 143 and UP 148) isolated from *Lotus corniculatus* rhizosphere suppress damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* in this legume (Bagnasco et al., 1998). The mechanisms involved in this action include the production of several antibiotics (De La Fuente et al., 1998). The successful development of a mixed pseudomonads-rhizobia inoculant requires the assessment of the dynamics of microbial populations in the rhizosphere, given that the introduction of antagonistic organisms to control root pathogens should not act against the diazotrophic bacteria.

The nodulation kinetics of *Mesorhizobium loti* B816 (commercial inoculant) on *L. corniculatus* was not affected by the presence of *P. fluorescens* UP61 or UP143. Nevertheless, with *P. fluorescens* UP148 a marked delay in nodule emergence was observed. The total number of nodules per plant was the same in the control (*M. loti* B816) and in all the co-inoculated treatments. None of the pseudomonads modified the aerial dry weight of plants compared to the control.

Root colonization patterns under growth chamber and field conditions in the systems *M. loti B816-L.corniculatus* and *Sinorhizobium meliloti* MCH3-alfalfa were studied using antibiotic-resistant mutants. Rhizospheric populations of either pseudomonads or rhizobia in *L. corniculatus* and alfalfa, were not negatively affected when both strains were inoculated together. The three *P. fluorescens* strains presented a similar colonization pattern in field assays up to 64 days after sowing, critical period for legumes establishment. No obvious differences in nodulation were detected among co-inoculated treatments and the control inoculated with *M. loti* B816.

Our results indicate that *P. fluorescens* strains isolated from *L. corniculatus* can colonize roots and survive in the rhizosphere of alfalfa in natural soil. This is an advantage for a wider use of these strains as biological control agents in legume diseases. The compatibility of these rhizospheric microorganisms offer a promising perspective for the future application of a mixed rhizobia-pseudomonads inoculant to increase forage legumes yield.

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EFFECTS OF ENVIRONMENTAL FACTORS ON THE GROWTH AND NITROGEN FIXATION OF *CASSIA MIMOSOIDES* VAR. *NOMAME*

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Present study aimed to clarify the effects of environmental factors on the growth and nitrogen fixation activity of *Cassia mimosoides* var. *nomame* which is a representative annual nodule plant in the dry and unfertile areas of Korea. Once the symbiosis was well established, the plants were subjected to water stress, defoliation and shading treatments. Plants were harvested at 14, 28 and 42 days after sowing.

Nodule formation in the roots of *Cassia mimosoides* var. *nomame* started on 28 days after sowing, and specific nitrogenase activity of nodule attained the maximum rate of 85 μM $C_2H_4 \cdot g$ fr wt nodule^{-1}·.h^{-1}on 42 days after sowing. The optimum temperature for the nodule activity was 30°C. Diurnal changes of nodule activity showed the maximum rate at 12:00 o'clock and the minimum rate during night.

The acetylene reduction activity (ARA) of dark treated plant decreased the activity by 85% in 1 day and 100% in 3 days after treatment. The ARA of completely defoliated plant decreased the activity 60% and 80% in 1 hour and 6 hours after treatment, respectively. The ARA of nodules with 50% defoliation decreased to 80% in 1 day and to 50% in 2 days.

The leaf water potential of the plant was -1.18 Mpa in 4 days after water stress treatment. When soil water potential decreased to -2.5 Mpa, the leaf water potential attained -2.2 Mpa and the ARA of nodules was 8 μM C_2H_4 g fr wt nodule^{-1}·h^{-1}. After water supply, the leaf water potential and the ARA of nodules were recovered in 1 day and 3 days, respectively.

When water restrained during flowering period, leaf and soil water potentials were -1.17 and -0.36 Mpa, respectively and the ARA resulted in 34 μM $C_2H_4 \cdot$ g fr wt . nodule^{-1}·h^{-1}The ARA was recovered rapidly by water supply. Leaf water potential of inoculated plot changed more rapidly than that of sterilized plot. The nitrogenase activity of *Cassia mimosoides* var. *nomame* seems to be more sensitive to the environmental restrictions such as shading, defoliation, drought, salts, heavy metals and oxygen stresses etc. than the other metabolic reactions.

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PEANUT INOCULATION IN A LOW FERTILITY SOIL

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There is considerable interest in improving nitrogen fixation in legumes to increase soil fertility. Peanut (*Arachis hypogaea* L.) is a very important legume grown in Argentina, with 98 % of its production concentrated in the province of Cordoba. Argentina is ranked as the second largest peanut exporter in the world. Inoculation of peanut is not a common practice in the country, but in low fertility soils the introduction of peanut microsymbionts may improve grain yield. This study was undertaken to determine the effect of peanut inoculation on the yield and nitrogen content in a low fertility soil.

Bacteria used in this study were TT002 (a native strain) and *Bradyrhizobium* SEMIA 6144 (recommended as inoculant). Both were spontaneous mutants resistant to streptomycin (200 and 40 µg/ml, respectively). Field study was conducted in 1998/1999 and the design was a randomized complete block with three treatments (TT002 or SEMIA 6144 inoculated seeds and uninoculated seeds). At growth stages R1 and R6 plants from a section of 1 m^2 in each plot were harvested. Nodule and plant dry weight, nodule number and occupancy (Somasegaran, Hoben, 1994) and nitrogenase activity (Hardy et al 1973) were determined. Soil and plant nitrogen content (Kjeldahl digestion) and yield seeds were also analyzed.

Table 1 shows that there were no differences in the symbiotic performance of plant from inoculated or uninoculated seeds. Yield seed was not affected by the treatment (Table 1). The nitrogen content determination revealed that the contribution of nitrogen fixation was by 80%, without influence of the inoculation. The nodule occupancy by the introduced strains was very low (SEMIA 6144: 7%, TT002: 8 %). In view of these results it is possible to assume that the introduced strains are less competitive than the native population. Further studies to select a more competitive strain to inoculate under this condition are necessary.

Table 1. Effect of peanut inoculation on symbiotic performance

Parameters	***Uninoculated***		***TT002***		***SEMIA 6144***	
	R1	**R6**	**R1**	**R6**	**R1**	**R6**
ARA(µmol ethylene/h/plant)	6.5	16.1	6.5	24.5	5.9	27.1
Nodule dry weight (mg)	107.9	570.0	103.0	501.2	132.2	505.3
Plant dry weight(g/plant)	4.3	24.8	4.2	30.6	5.0	28.3
Nodule number/plant	116.1	762.3	131.7	642.6	136.5	789.1
Seed Yield (kg/ha)	1924		1939		2041	

Data represent the averages of three replicates. Duncan test P=0.05

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DISTRIBUTION OF A POPULATION OF *RHIZOBIUM LEGUMINOSARUM* BV *TRIFOLII* AMONG DIFFERENT SIZE CLASSES OF SOIL AGGREGATES

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1. Introduction

In this study we have been interested in the distribution in the soil matrix of a particular member of the soil community, namely *Rhizobium leguminosarum* bv *trifolii.* Despite being recognized primarily for its ability to form symbiotic associations with leguminous plants, this species is a bona fide soil saprophyte. In addition to that, the existence of well documented methods such as direct counts with fluorescent antibodies, makes *Rhizobium* a singular microorganism for this kind of study, in comparison to other members of the soil microflora. Our objective was to gain novel insights into the spatial arrangement between soil bacteria and the soil matrix by examining the distribution across soil aggregates of *Rhizobium leguninosarum* bv *trifolli*, in the presence and absence of its legume host plant.

2. Materials and Methods

A combination of the most probable number (MPN) technique and immunofluorescence direct count (IFDC) microscopy was used to examine the effects of three winter cover crop treatments on the distribution of a soil population of *Rhizobium leguminosarum* bv *trifolii* across different size classes of soil aggregates (<0.25, 0.25 to 0.5, 0.5 to 1.0, 1.0 to 2.0, and 2.0 to 5.0 mm). The aggregates were prepared from a Willamete silt loam soil (0-20 cm) immediately after harvest of broccoli (September 1995) and before planting and after harvest of sweet corn (June and September 1996, respectively). The summer crops were grown in soil that had been either fallowed or planted with a cover crop of red clover, *Trifolium pratense* (legume) or Celia triticale (cereal) from September to April. The experimental design was a complete randomized block with four replications of each treatment.

3. Results

The *Rhizobium* soil population was heterogeneously distributed across the different size classes of soil aggregates and the distribution was influenced by cover crop treatment and sampling time. On both September samplings, the smallest size class of aggregates (<0.25 mm) recovered from the red clover plots carried between 30 and 70% of the total nodulating *R. leguminosarum* population, as estimated by the MPN procedure, while the same aggregate size class from the June sampling carried only ~6% of the population. In June, IFDC microscopy revealed that the 1.0-2.0 mm size class of aggregates from the red clover treatment carried a significantly greater population density of the successful nodule-occupying serotype, AR18, than did the aggregate size classes of <0.5 mm and 2.0 to 5.0 mm. In September, however, the population profile of AR18 had shifted such that the density was significantly greater in the 0.25–0.50 mm size class than in aggregates of <0.25 mm and > 1.0 mm. The populations of two other *Rhizobium* serotypes (AR6 and AS36) followed the same trends of distribution in the June and September samplings. These data indicate the existence of structural microsites that vary in their suitabilities to support growth and protection of bacteria and that are influenced by the presence and type of plant grown in this soil.

IMPACT OF SOYABEAN BNF ON THE RURAL POOR: EXPERIENCES FROM ZIMBABWE

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Most grant proposals for legume biological N fixation are justified on potential benefits for the poor developing countries but often result in publications while the poor remain hungry and malnourished. Against a background of escalating mineral N fertiliser prices, harnessing biological nitrogen fixation is obligatory for African agriculture to arrest deteriorating soil fertility and bolster food security of the poor smallholder farmers (Mpepereki & Makonese, 1998).

Farmer-managed trials and demonstrations were conducted to explore the performance of 4 soyabean varieties (2 promiscuous - Magoye and Local and 2 specific - Roan and Nyala) with and without rhizobial inoculation in a wide range of smallholder cropping environments. Extension agents and farmers were trained in use of soyabean inoculants. Women were trained in processing and utilisation of soyabean as food at family level. The technology was evaluated during numerous field discussion days with farmers and extension workers. Mobilisation was improved by involving local leaders (chiefs, councillors, MPs). Farmers were assisted to access inputs (seed, fertilisers and inoculants) and viable markets for their soyabean crops.

Our research showed that soyabean can be grown under smallholder conditions with yields ranging from 0.8 - 3.5 t/ha, exploding a widely held myth that soyabean does not do well under smallholder management. Soyabean stover yields ranged from 2 - 4 t/ha (30 - 60 kgN/ha) and when incorporated gave maize yields of 2 - 4 t/ha compared to 0.4 - 1 t/ha for maize after maize. For poor farmers, these organic and N inputs represent significant savings in N-fertilizer costs. Farmers sold 60% of their produce realising significant cash income, that they have used to purchase agricultural inputs, implements and meeting family budgetary needs. About 25% of the crop has been retained for food, representing 80 - 350 kg of dietary protein per family per year and contributing significantly to food security of the vulnerable poor. About 15% of harvested soyabean is retained for seed which is the most expensive input. Promiscuous varieties produced the largest quantities of biomass and had the added advantage of not requiring rhizobial inoculants.

Over the 3 seasons (1996 – 1999) there was a phenomenal increase in the number of participating farmers from 50 to >6000. Soyabean deliveries by smallholder farmers to the formal market rose from 18 tons in 1996/97 to > 1000 tons in 1998/99 with total earnings of over ZW$8 million. We have demonstrated potential soyabean BNF to enhance food security, per capita income and improve soil fertility for sustainable crop production by poor smallholder farmers. Efforts are now underway to extend the technology to more farmers. Adaptive research is being conducted to underpin the soyabean extension program. Our experience in Zimbabwe is that there is great potential to use soyabean BNF as a vehicle for socio-economic empowerment for the rural poor.

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CONTRIBUTIONS OF BELOW-GROUND LEGUME N TO THE N BUDGETS OF CROP ROTATIONS

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1. Introduction

Calculations of N_2 fixed by legumes are normally based solely upon shoot-based measures of fixed N, with the below-ground contribution of N ignored. The aim of this study was to adapt a ^{15}N shoot labelling technique to quantify the below-ground fraction of legume N in the filed.

2. Materials and Methods

A field experiment (8 microplot, 0.5 x 0.64 m) with chickpea and faba bean was conducted at the Breeza long-term experimental site in the northern New South Wales, Australia. All the plants within microplot were fed 0.2 ml of 0.5% ^{15}N urea (99% atom% ^{15}N) on five occasions during vegetative and early reproductive growth. The microplots were harvested just prior to physiological maturity. Shoots, intact roots and all soil to 25 cm depth (8 soil corse of 25-45 cm) were recovered for dry matter, %N and ^{15}N analysis.

3. Results and discussion

The ^{15}N enrichment of all plant and soil samples was measurably greater than natural abundance. Recoveries of ^{15}N were >80% of the ^{15}N supplied to the plant shoots during growth. Calculations using the ^{15}N excess of the recovered roots and the ^{15}N enrichment of soil suggested that 24 and 76% of total crop N was associated with the roots of faba bean and chickpea, respectively (Table 1). The below-ground value for faba bean (equivalent to 41 kg N/ha) lay similar to previous measurements using ^{15}N-based techniques (Russell and Fillery 1996; Rochester *et al.* 1998). However, the value derived for chickpea roots seemed excessively high because of very low ^{15}N content in the recovered roots, and a very high in the 0-25 cm soil component (Table 1).

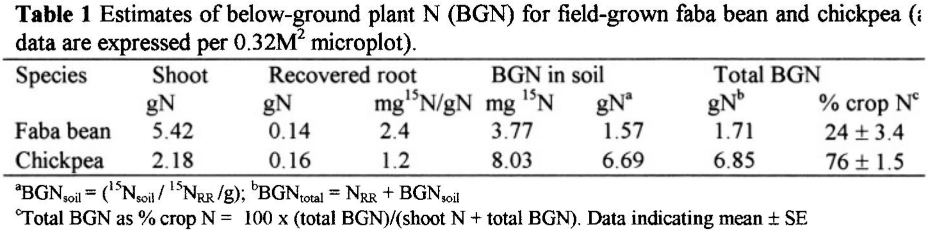

Table 1 Estimates of below-ground plant N (BGN) for field-grown faba bean and chickpea (
data are expressed per $0.32M^2$ microplot).

Species	Shoot	Recovered root		BGN in soil		Total BGN	
	gN	gN	mg^{15}N/gN	mg ^{15}N	gN[a]	gN[b]	% crop N[c]
Faba bean	5.42	0.14	2.4	3.77	1.57	1.71	24 ± 3.4
Chickpea	2.18	0.16	1.2	8.03	6.69	6.85	76 ± 1.5

[a] $BGN_{soil} = (^{15}N_{soil} / ^{15}N_{RR} /g)$; [b] $BGN_{total} = N_{RR} + BGN_{soil}$

[c] Total BGN as % crop N = 100 x (total BGN)/(shoot N + total BGN). Data indicating mean ± SE

4. Conclusion

Shoot-labelling techniques have been successfully adapted for quantifying below-ground N in legume species. The field studies suggested that 24% or more of total legume N was associated with, or derived from roots. The value of legume rotations have been greatly underestimated in past calculations because this below-ground contribution of N has previously been ignored.

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STEM NODULES MORPHOLOGY OF *DISCOLOBIUM LEPTOPHYLLUM*

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Discolobium is a native stem and root-nodulating forage legume from the Pantanal ecosystem, west region of Brazil (Mato-Grosso state) (Loureiro et al., 1994). The genus *Discolobium* has its native habitat in the swamp regions and flooded areas. Stem nodules as well as root nodules are able to fix nitrogen and they show peculiar formation and structure in *Discolobium* species described. The stem nodules of *D. leptophyllum* are obligately aquatic, requiring permanent submergence.
The material was prepared for optical microscopy according to James et al. (1992). Samples of stem nodules were fixed in 2,5% glutaraldehyde in 50mM phosphate buffer at pH 7,0. The samples were washed in buffer and dehydrated in an ethanol series. After 7 days infiltration, the nodule pieces were embedded in LR White resin (London Resin Company) by heat polymerization at 60°C. Ultrathin sections (1-2μm) were cut with ultra-microtome, stained in toluidine blue 0,1% (pH 6,8) and observated in optical microscope

The new species *D. leptophyllum* also presented stem nodules with vascular bundle directly connected to the stem vascular bundle. The vascular system from the stem branched throughout the nodule, penetrating the infected tissue within finger-like ingrowths of the cortex. The nodules are aeschinomenoid type, without uninfected cells within infected tissue. The cortex of stem nodules shows different cells layers. The inner cortex points out cells layers beside a infected zone contained cells small, closely packed with few intercellular spaces and numerous large amyloplasts.

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SYMBIOTIC N_2 FIXATION BY FABA BEAN (*VICIA FABA* L.) IN A GYSPUM-TREATED SODIC SOIL

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Sodicity is a significant economic and environmental problem for many Australian soils used for growing grain legumes. The most common means to ameliorate these soils is through the use of gypsum. However, little is known about the impact of gypsum applications on proportional symbiotic performance (P_{fix}) of grain legumes. A field experiment conducted at Nhill in Victoria used faba beans (*Vicia faba* var. Fiord). Four rates of phospho-gypsum (0, 2.5, 5, 10 t ha^{-1}) were surface applied two years before the experiment was conducted. At crop emergence, unconfined micro-plots (2.9 m x 2.4 m) were randomly located within the main plots. $^{15}N\text{-}NH_4Cl$ (15.3 atom %) was applied at a rate of 8 kg N ha^{-1}. Soil was sampled to 15cm at 48, 62, 76, 90, 103, 121, 143, 158 and 183 days after sowing to determine ^{15}N atom % excess in soil mineral N pools (NO_3^- and NH_4^+). Plants were also sampled at these times to determine N and ^{15}N atom % excess. Symbiotic N_2 fixation was estimated using two ^{15}N dilution modelling methods - yield-dependent (Smith *et al.,* 1992) and yield-independent (Chalk *et al.,* 1996) - as well as a reference plant method using canola. Both models use the mean pool abundance of total mineral ^{15}N as reference.

At physiological maturity, P_{fix} values estimated by all three methods, were highest in 5 t ha^{-1} treatments. There were no significant differences between 0 and 2.5 t ha^{-1} treatments, and adverse effects by gypsum applied at 10 t ha^{-1} except in the case of the Y-I model (Table 1). Estimates of P_{fix} using the modelling methods were sensitive to the soil ^{15}N pool used as the reference. Estimates of P_{fix} were improved when the decline of ^{15}N in the total inorganic pool was used as a reference rather than the decline of the $^{15}N\text{-}NO_3^-$ pool alone, because of high concentrations of $^{15}N\text{-}NH_4^+$ that remained in the soil until anthesis. Seasonal patterns of P_{fix} were similar between the reference plant and yield-dependent model but the yield-independent model appeared to over estimate P_{fix}. Total N accumulation was significantly increased by the gypsum treatments compared with the controls with the effect being highest in 10 t ha^{-1} plots. Grain production per plant was also affected by the gypsum rates with 3.2, 4.2, 4.7, and 4.4 g $plants^{-1}$ being recorded for the 0, 2.5, 5 and 10 t ha^{-1} treatments, respectively. Thus, gypsum application to ameliorate sodic soils increased biological N_2 fixation by grain legume crops and the total amount of N uptake. There were reasonable agreements between N_2 fixation estimated by the traditional reference plant method and by the yield-dependent soil ^{15}N decline model.

Table 1. N_2 fixation response to gypsum treatments at physiological maturity,

Gypsum	P_{fix} (%)*			Total N
(t ha^{-1})	Y-I*	Y-D*	Ref	(g N m^{-2})
0	74	66	65	19.3
2.5	81	55	64	18.8
5	82	88	69	24.8
10	82	46	68	26.6

*Y-I and Y-D are yield independent and dependent models, respectively; Ref, reference plant technique.

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PERSISTENCE BEHAVIOUR OF GENETICALLY MODIFIED RHIZOBIA IN ITALIAN SOILS

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An eight-year study to evaluate the ecological outcome of open field-released genetically modified *Rhizobium leguminosarum* bv. *Viciae* stemmed from the European biosafety and risk assessment projects and focused on the effect of genetic modifications on bacterial environmental fitness as well as on their ecological impact. Appropriate marker genes, beta-galactosidase and mercury resistance, were used as strain tags. To compare locus-dependent stability, the introduced genes were carried either on an introduced plasmid or homogenotized into the chromosome. In addition, the behaviour of an allochthonous strain versus an autochthonous one that was dominant in the release site were compared. Upon authorization, the three GM rhizobia were released as field inoculants in the presence of their pea host by liquid inoculation at about $5x10^6$ cfu per seed. They were released at the agricultural experimental station of the University of Padova. Bacteria were monitored in soil, rhizosphere and nodules. The parental strain 1003, from which the GMMs were derived, had been isolated in northern Europe and propagated for a considerable time in the laboratory environment. It was therefore allochtonous, with respect to the release site. This condition, along with a possible fitness reduction due to extended laboratory maintenance, may have contributed to its very poor persistance. In parallel, the possible impact on resident biota was measured by plate counts of total aerobic culturable bacteria, spore-forming bacteria, micromngi, streptomycetes, fluorescent pseudomonads, and total rhizobia. No significant numerical perturbations were detected.

The experience gained by field release of an allochtonous strain prompted the question of how a competitive, indigenous *Rhizobium* engineered with the same genetic modification might have fared. To address this question, we analyzed a set of RAPD fingerprinting data of >250 nodule occupants from the first release site. The *R. leguminosarum* bv. *viciae* natural local population showed more than 30 different RAPD profiles, some of which were present in many nodules. This led us to identify the profile of the dominant indigenous strain which occupied over half of the nodules. A spontaneous rifampicin-resistant derivative of this strain was genetically modified by introducing plasmid pDG3, yielding the equivalent of strain 1110 used in the first release, but now in a strain native to the chosen habitat and successful on its symbiotic hosts. The strain was named 1114. The performance of this GMM was up to the standards of its parent whose competitive traits were maintained in terms of nodulation and persistence in the habitat. Despite active pea colonization and infection by strain 1114, again the resident microbiota showed no significant alterations as determined by plate counting, indicating that a GMM extensively invading its environment, does not necessarily alter the soil populations. When comparing plots where strain 1114 was released as a pea inoculant with those where it was released in soil without plants, the initial plant effect was clearly supportive. The strain in bare soil showed lower values and, even when plants were added later, it could never be recovered in numbers comparable to those found in plots where pea was always present. Eventually, after four years, the number in the bare soil plot fell below the detection limit. A striking result was observed in bare soil amended with lactose, where the GMM persisted at a detectable level ($>10^3$) for over 4 years. In conclusion, long-term strain persistance was more positively affected by the initial exposure to a supporting nutritional advantage in a saprophytic state than was conferred by the development of a symbiotic interaction with the host plant.

APPLICATION OF THE "^{15}N NATURAL ABUNDANCE METHOD" TO ESTIMATE BIOLOGICAL NITROGEN FIXATION BY LEGUMINOUS TREES IN FOREST STANDS OF THE EASTERN AMAZON

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Biological nitrogen fixation (BNF) in tropical moist forests is difficult to quantify, due to the lack of suitable techniques allowing accurate estimation of this input. Aim of this research was to find out whether the ^{15}N natural abundance method (^{15}NNAM) could be applied for quantifying BNF in the fallow vegetation of the slash and burn system in the NE Amazon, Brazil.

Elaborate surveys in 5 to 6 year old stands were conducted to assess the spatial and temporal variations of ^{15}N in tree species, 3 of them leguminous. A-plants were cultivated in the greenhouse. Three selection criteria were used to identify the most suitable reference plants:
-The similarity in temporal δ^{15}N-pattern in young leaves (varying replications)
-The greatest dissimilarity in δ^{15}N with those of the legumes and the lowest δ^{15}N spatial variance (6 stands, 5 replication/species)
-The coincidence of δ^{15}N-pattern amongst different leaf ages between reference plant and legumes.
A cluster analysis was conducted with the data of the temporal and spatial surveys

Young leaves had δ^{15}N-values between -4 and +6 with an average of -0.8, but with a high variability between stands. Leguminous plants showed a closer range of +1.1 and -0.9 with less variability among the six stands, suggesting a certain site independence probably due to BNF.

Selection of reference plants:
Temporal variability: For the most species a drop in δ^{15}N indicated the start of the rainy season, but with time δ^{15}N returned to the values measured before. Those species, which did not return to the previous δ^{15}N and/or demonstrated only a small or no drop in δ^{15}N were considered not suitable.
Spatial variability: *Banara guianensis* and *Lacistema pubescens* showed δ^{15}N-values too close to the legumes (difference<1 δ^{15}N-unit). *Vismia guianensis* showed an exceptionally high site-to-site variation (positive and negative δ^{15}N).
Translocation behavior: *Vismia guianensis* showed the same δ^{15}N difference between young and old leaves as the legumes. *Tapirira guianensis* did not show significant differences. *Lacistema pubescens* and *Banara guianensis* had very large differences and a very high variability.

Cluster analysis: Three groups in agreement with the results of our selection procedure were formed. 1) Legumes (high %N in the leaves, δ^{15}N of the leaves near 0), 2) the selected reference plant (negative δ^{15}N-values high %N), 3) Species with highly variable, often positive δ^{15}N-values and low N-content.

Using *Tapirira guianensis, Vismia guianensis* and *Mabea angustifolia* as the most suitable reference species and the a-plants for discrimination correction, the BNF of *Abarema jupunba, Inga thibaudiana* and *Inga heterophylla* were calculated. Between 32% and 75 % of the nitrogen in young leaves was apparently derived from the atmosphere (%Ndfa), with a standard error (after Shearer and Kohl, 1988) between 11 and 39% of the estimated Ndfa (average of 22%).

The results showed that the application of the ^{15}NNAM in a complex natural vegetation calls for extensive surveying in order to identify appropriate reference plants. As a result, estimates of %Ndfa are semi-quantitative at best. But these data also illustrate that the native legumes of the investigated forests could play an important role in restoring the nitrogen lost due to agricultural practices.

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^{15}N DETERMINATION OF THE CONTRIBUTION OF BNF TO THE FORAGE LEGUME *ARACHIS PINTOI*

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Arachis pintoi is a forage legume which is increasingly being utilised in tropical pastures due to its capacity to support high grazing pressure and high beef and dairy cattle production. Under grazing in a mixed sward with star grass (*Cynodon nlemfuensis*) in Costa Rica the proportion of N derived from BNF was estimated to be approximately 80 % using the isotope dilution technique using ^{15}N enriched fertiliser. Using the same technique on a 3-year-old protein bank of this legume BNF was found to be insignificant, probably due to the build up of N under the legume. In mixed pastures with *Brachiaria dictyoneura* in the South of Bahia (Brazil) similar high proportions of N derived from BNF were registered using the ^{15}N natural abundance technique.

PUERARIA PHASEOLOIDES AS A COVER CROP IN AGROFORESTRY SYSTEMS IN THE HUMID TROPICS : GROWTH, NUTRITION AND BIOLOGICAL N_2 FIXATION

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Apart from effects on soil protection, legume cover crops have are important for nutrient cycling in fruit production systems in the humid tropics. Our objective was to analyse the biomass production and nutrition of *Pueraria phaseoloides* as a cover crop grown in three different agroforestry systems (System 1: *Theobroma grandiflorum, Bactris gasipaes* and *Hevea* spp; System 2: *Theobroma grandiflorum, Bactris gasipaes, Bertholletia excelsa* and *Bixa orellana*, System 3: *Theobroma grandiflorum, Hevea* spp, *Cocus nucifera* and *Citrus sinensis*) at two levels of fertilisation (100 % + additional dose of P and 30 % of fertilisation recommended to individual useful plants), and its potential to contribute to the N stocks of the cropping system by biological N_2 fixation (BNF). The study was conducted on experimental plots with the dimensions of 32 by 48 m using three replications planted in 1993. The site's soil is a Xanthic Ferrasol (FAO/UNESCO). For estimating the above-ground biomass, the litter and plant nutrition of *Pueraria,* five sub-samples of $0.25\ m^2$ were collected from the area between the tree rows in each plot in June 1998. The samples were dried at 70°C for 48 hours and nutrient contents (N, P, S, K, Ca, Mg, Fe, Mn, Zn and Cu) were measured. BNF was determined in both the dry and rainy season in system 1 (100% fertilisation) using ^{15}N isotope dilution. Three different non-fixing reference plants were used which had similar growth characteristics (*Rolandra fruticosa, Maieta* sp, and a Cyperaceae species). Ammonium sulfate with 10 atom% ^{15}N excess was applied at a rate of $50\ mg\ m^{-2}$ in microplots of $1\ m^2$. Statistical analyses were done with ANOVA using a randomized complete block design.

The fertilisation levels had no influence on aboveground biomass, litter stocks and foliar N, P and micronutrient contents of *Pueraria* ($P>0.05$). Only K and Ca contents in the biomass were influenced by fertilisation indicating that nutrient applications to the trees can improve cover crop nutrition. As a consequence, a positive correlation was found between the aboveground biomass and the K contents ($R=0.51$; $P< 0.03$). Additionally, N and P contents ($R=0.63$; $P<0.005$) as well as N and Mg contents ($R=0.56$; $P< 0.01$) were significantly related to each other. However, the type of cropping systems was the main factor controlling growth and the nutritional status of *Pueraria.* The aboveground biomass and litter of *Pueraria* ranged from 2.2 to 6.1 and from 2.7 to $7.7\ Mg\ ha^{-1}$, respectively. The highest aboveground biomass and litter production was found in systems 3 with the most scattered plant distribution. The N content of *Pueraria* reached $57\text{-}177\ Kg\ ha^{-1}$ in the aboveground biomass with similar values in the litter of $48\text{-}165\ Kg\ ha^{-1}$. In contrast, the amounts of Ca in the litter were enriched in comparison to the biomass, whereas K was depleted due to rapid leaching from decomposing litter. With the exception of K, the nutrients stocks in *Pueraria* litter were more than twice as high as the nutrients applied by fertilisation. Consequently, nutrient turnover of *Pueraria* was an important process in the nutrient cycling of the fruit tree production system. The N in *Pueraria* derived from biological N_2 fixation estimated by ^{15}N enrichment reached values from 9 to 45 % and was higher throughout the wet than the dry season. Thus, $32\text{-}158\ Kg\ N\ ha^{-1}$ of the biomass N were derived from atmospheric N_2, exceeding the amounts of N added by fertilisation. The large nutrient uptake and return through litter indicated a rapid nutrient cycling at the plant-topsoil interface, thus reducing leaching and keeping nutrients available. The considerable amount of biologically fixed N_2 was a relevant and important addition to the N pool of the cropping systems.

ACTIVITIES OF THE EUROPEAN COMMISSION IN THE FIELD OF SCIENTIFIC & TECHNOLOGICAL CO-OPERATION WITH DEVELOPING COUNTRIES ON BNF

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Agricultural and environmental research for development are key issues for scientific and technological (S&T) co-operation of the European Union (EU) with Developing Countries (DCs). Biological nitrogen fixation (BNF) as a particular important subject has been part of the EU S&T co-operation activities since its beginning. This co-operation has developed continuously during the last 17 years.

1. INternational CO-operation (INCO) a horizontal programme and BNF

INternational CO-operation –DEVelopment oriented research (INCO-DEV) presents the current activity in the 5th Framework Programme on Science, Research and Technological Demonstration (FP-5) under which research co-operation with DCs on BNF can be carried out. It is based on equal footing between partners from the EU and DCs in fields of common interest for countries in a given region. The activity privileges an interdisciplinary approach and distinguishes between research oriented towards policies, systems and technological tools. The considered research topics are announced in calls for proposals.

The minimum consortium of partners consists of 2 partners from 2 different DCs in a region, together with 2 partners from 2 different EU Member States. In case of China or India 2 partners from two different regions in one of these countries are eligible as well. A partner from a country associated to FP-5, may qualify like a partner from the EU. The DC regions considered are Sub-Saharan Africa, Caribbean and Pacific (ACP) countries, Latin America, Asia and Mediterranean Partner Countries (MPCs).

Basically all issues related to BNF as an instrument for crop improvement, from the point of view of agro-economy and environmental protection, are covered (biotechnology, inoculation, agro-forestry, inter-cropping, farming systems, crop rotation, microbiology as well as nitrogen losses and pollution).

The application of technological tools such as breeding, integrated pest management and agronomic techniques for improving soil fertility in the calls is limited to certain legumes, staple food crops and cash crops of particular interest for different regions (please check details also with the work programme of the call). These are for the 1st call in 1999:

ACP-countries:

Legumes: *Vignia unguiculata, Phaseolus vulgaris, Cajanus cajan.*
Staple food crops: rice, maize, millet (*Pennisetum typhoides*), sorghum, yam, cassava, plantain.
All under-utilised or minor grain legumes and staple food crops.

Cash crops: to be considered in a subsequent call.

Asia:

Legumes: *Cajanus cajan.*
Staple food crops: rice, maize, cassava, plantain, sweet potato.
All under-utilised or minor grain legumes and staple food crops.

Cash crops: to be considered in a subsequent call.

Latin America:

Legumes: *Phaseolus vulgaris.*
Staple food crops: maize, wheat, cassava, potato (*Solanum tuberosum*), plantain.
All under-utilised or minor grain legumes and staple food crops.

Cash crops: to be considered in a subsequent call.

MPCs:

Legumes: *Pisum sativum, Vicia faba, Phaseolus vulgaris, Cicer arietinum.*
Staple food crops: barley, wheat, rice, potato *(Solarnum tuberosum).*
All under-utilised or minor grain legumes and staple food crops.

Cash crops: to be considered in a subsequent call.

Subsequent calls will be launched in 2000 and 2001.

2. Thematic programmes with key actions and generic activities for research on BNF

Beside the co-operation within the INCO-DEV activity, there is within FP-5 for the first time, the possibility for participation of researchers from DCs in the main thematic programmes. Partners from associated countries have full access like partners from EU Member States. There are also possibilities for participation of partners from other third countries on a project by project basis. Prior conditions for the participation of researchers from third countries are:

- ☐ The existence of an RTD co-operation agreement for FP-5; or
- ☐ Considerations related to the interest of the EU in co-operation with third countries from the Mediterranean and MPCs or international organisations, (in particular, needs of EU policy, effect on employment in the EU, improvement of sustainable development as well as the quality of life in the community, strengthening of the international competitiveness of the EU-industry); or
- ☐ Additional added value for European research, (e.g. top researchers, access to exceptional resources not available in the EU, probability for new market access for European participants, etc.).

The main thematic programmes are structured in key actions focussing on different research topics dependent from the priorities set. Beside these key actions, every main thematic programme contains a group of generic activities, covering a wider range of subjects.

The thematic programme Quality of Life and Management of Living Resources (LIFE) has several activities where proposals on BNF can be submitted. Key action 3 “Cell-factory” focuses on the investigation of mechanisms of cells to use them for the improvement of the quality of life and management of environmental problems. Key action 5 Sustainable Agriculture, Fisheries and Forestry, as well as Rural Development including Mountain Areas as a research instrument to support the Common Agricultural Policy (CAP) of the EU.

The thematic programme Energy, Environment and Sustainable Development (EESD) contains several activities to fund proposals in the field of BNF. Key action 2 focuses on global change, climate and biodiversity and deals also with the better comprehension of terrestrial, limnological and marine ecosystems thereby covering the nitrogen cycle.

Information about European research and FP-5 is also available at the Cordis – server (www.cordis.lu).

SECTION XII

SOIL RECLAMATION AND REMEDIATION

THE IMPORTANCE OF BIOLOGICAL NITROGEN FIXATION ON LAND REHABILITATION

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1. Introduction

More than 50% of all cultivated tropical soils are considered to be already at a higher level of degradation. That is generally a consequence of loss of soil organic matter and its benefits as a source of nutrients and as a soil conditioner. The addition of large amounts of organic compost or surface soil has been extensively used to rehabilitate degraded soils, but it is expensive and not affordable to most farmers. Also, surface soil is seldom available since its removal causes ecological damages to the donor site. Diazotrophic micro-organisms, free living, associated or in symbioses with plants, are the first colonisers of nitrogen-deprived soil. The presence of mycorrhizae increases the plants efficiency in collecting water and nutrients from the soil, especially phosphorus. Thus, the symbiosis of plant species with diazotrophic bacteria and mycorrhizal fungi allows colonisation of substrates deprived of organic matter, where acidity, aluminium and/or manganese toxicity and water stress are prevalent as indicated in Figure 1. Most of the tropical legume species are highly dependent on mycorrhizal (Figure 2).

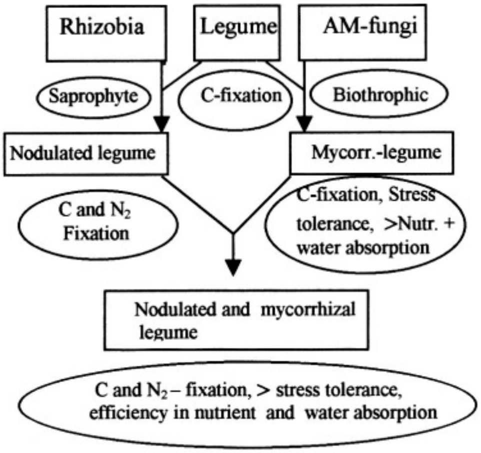
Rhizobia
Legume
AM-fungi
Saprophyte
C-fixation
Biothrophic
Nodulated legume
Mycorr.-legume
C and N_2 Fixation
C-fixation, Stress tolerance, >Nutr. + water absorption
Nodulated and mycorrhizal legume
C and N_2 – fixation, > stress tolerance, efficiency in nutrient and water absorption

Figure 1- Schematic representation of trophic Levels on the symbiosis of nodulating legumes With rhizobia and mycorrhizal fungi (Modified from Souza, Silva 1997).

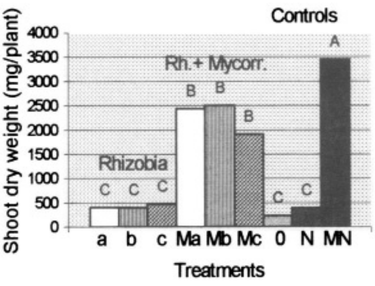

Figure 2. Shoot dry weight of *Anadenanthera peregrina* inoculated with rhizobia (a=BR 9001, b=BR 9004, c=BR 4812), mixture of arbuscular micorrhizae fungi (M) compared with a control without (O) or with nitrogen (N). (J. Schiavo, S. M. de Faria, unpublished).

2. Nitrogen and Phosphorus Supply

Biological Nitrogen Fixation (BNF) is the natural source of nitrogen for land reclamation. Multipurpose nodulating legume trees supply nitrogen and other benefits such as wood, fire

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wood, forage, honey, fruit and water storage.(Budowski, Russo 1997). The acidity generated on the root surfaces of plants supplied by BNF (Raven et al 1990) increases P availability from rock phosphate (Silva et al 1985) supplying P to plants for long periods even in soils with high P adsorption capacity. Thus, nodulated and mycorrhized legume species, with addition of rock phosphate, may be successful in colonising exposed subsoil and substrates devoid of organic matter. This may be possible by using nodulated and mycorrhizal seedlings or by hydro-sowing seeds together with rhizobia and mycorrhizal fungi in unconsolidated substrates of species adapted to oligotrophic conditions (Franco et al 1995). Although, under tropical conditions, the legume does not change significantly oil fertility (Dias et al 1995), succession experiments on rehabilitated lands show that the benefit of colonisation with nitrogen-fixing species both by the increase in biomass and the exuberant development of the succeeding species (Campello 1999).

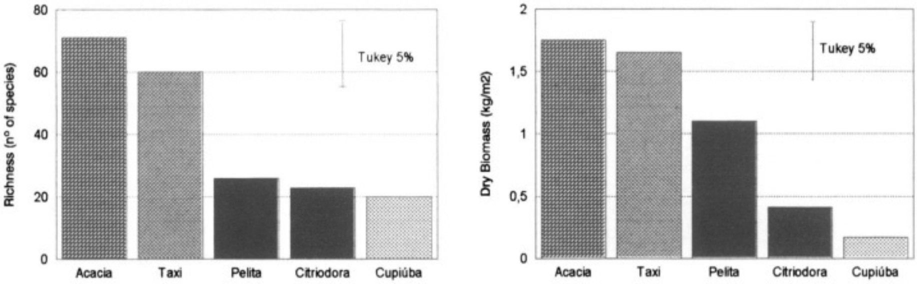

Figura 3 -Plant species richness and biomass of natural regeneration in succession of N_2–fixing (*Acacia mangium*, *Sclerolobium paniculatum* –taxi) and non legume tree species (*Eucalyptus pellita*, *E. citriodora* and *Goupia glabra* – cupiúba). (Campello, 1999).

These experiments, conducted in large areas of soils devoid of organic matter, may be used as a model to show the great potential of BNF in cropping systems, far beyond the present contribution and on reclamation of large areas of degraded soils such as found in the tropics.

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SOIL BIOTESTS AND PHYTOREMEDIATION WITH THE LEGUME-*RHIZOBIUM* SYMBIOSIS

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1. Soil Biotests

Compared to biotests for groundwater and surface water quality, the number of accepted and sensitive biotests for soil contamination is rather limited. Growth effects on a number of agricultural plants such as *Avena sativa, Brassica rapa* or *Phaseolus aureus* are used (ISO/DIS, 1995) or effects on root growth (ISO, 1993). In general, plants are suitable as bioindicators of soil health, due to their intensive root system and the large contact area between the soil and the biomonitoring organisms (Pandolfini et al., 1997). The basis is a long tradition of using plants to indicate specific soil conditions such as *Salicornia* species for salt affected soils, *Teesdalia nudicaulis* for acid soils and *Mercurialis annua* for alkaline soils. Well studied is the use of sensitive plant species against heavy metal contamination and the colonization of these areas by metal tolerant populations (Verkleij, 1993). As specific biochemical markers, phytochelatins, specific enzyme activities such as peroxidase activity and the polyamines have been used. Changes in microbial populations and activities are another area of bioindicators of soil health (Roper, Ophel-Keller, 1997). Carbon transformations and nitrogen transformations are widely used, but not very specific, for they react against almost any other environmental stress on the microorganisms such as temperature, soil pH and water conditions.

Other bioindicator systems use extrapolation in ecosystems, as the factor method (OECD, 1998). The ratio of PEC (Predicted Environmental Concentration) divided by PNEC (Predicted No Effect Concentration) larger than 1 is considered as an environmental risk (Mathes, 1997). Also for other methods the no observed effect concentration is of central importance. Therefore the development of a more sensitive biotest than the established systems is very important for risk assessment.

2. The *Rhizobium*-Legume Symbiosis as a New Sensitive Biotest

During the last years the methodology and application of the *Rhizobium-legume* symbiosis as a new sensitive biotest has been developed towards heavy metals and organic contaminants (Wetzel et al., 1991; Wetzel et al., 1994; Wetzel et al., 1995; Wetzel, Werner, 1995; Wetzel, 1998; Neumann et al., 1998). Table 1 summarizes data of the significantly high sensitivity of nodulation, compared to shoot growth and root growth for most of the substances tested.

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Table 1. EC50 for nodulation, shoot growth and root growth in the system *Medicago sativa/Sinorhizobium meliloti*

Test compound	Nodulation	Shoot growth	Root growth
	EC50 mg l^{-1} and µg . cm^{-2} (*)		
Cadmium iodide	0.5	10.4	72.9
Cadmium chloride	1.1	19.6	4
Cadmium acetate	2.5	17.4	22.6
Sodium (meta) arsenate	0.3	93.9	82.4
Disodium hydrogen arsenate (V)	1.7	248.7	218,1
Arsenic pentoxide	4.2	157,6	143,2
Atrazine	0.13	0.15	0.09
Lindane	6.7	20	7.7
Fluoranthene*	2.5	> 35	> 35
Phenanthrene*	5.4	28	27
Fluorene*	7.1	21	22
Acenaphthene*	20.0	52.5	50.8

For cadmium and arsenic compounds the nodulation biotest is more sensitive by a factor of 10 to 100 compared to shoot growth and root growth. Against organic contaminants such as fluoranthene and acenaphthen the nodulation biotest is more sensitive by a factor of 2 to 10. A factor of 10 of higher sensitivity is a very significant progress in evaluating the potential risk in the food chain from the soil to the plant and to humans and animals.

3. Gene expression in the symbiotic system modulated by the xenobiotics cadmium and fluoranthene

Using mRNA differential display techniques in the symbiotic test system affected by cadmium and fluoranthene, 37 differentially displayed transcripts were detected. Two of them called DDMs1 and DDMs2 were confirmed by northern hybridization, regulated by the presence of the two xenobiotics (Neumann, Werner, submitted for publication). Transcription of DDMs1 was highly affected by the cadmium concentrations with an EC50 of 5,9 µM. This is almost identical to the EC50 found for nodulation. Sequence analysis of DDMs1 gave a significant identity to a hypothetical protein from *Arabidopsis thaliana* with a high similarity to a copper transporting ATPase. DDMs2 has a strong similarity (82% identity) to the cytoplasmatic 60S ribosomal protein LI8 from *Arabidopsis thaliana.*

4. Phytoremediation

The concept of phytoremediation has gained increasing general interest in the last years (Cunningham, Ow, 1996; Boyajian, Carreira, 1997; Salt et al., 1998). Phytoremediation is the use of plants to reduce pollutants in the soil by enrichment in plant material and removal of these plant parts. The application of phytoremediation is especially attractive when hyperaccumulating species for different heavy metals are used, such as *Psychotria douarrei* for nickel, *Thlaspi caerulescens* for cadmium, *Ipomoea alpina* for copper or *Macadamia neurophylla* for manganese. From a joint study at the Semipalatinsk Test Site, very interesting new results have been found for radionuclide accumulation in specific plant species from this region. In the legume *Glycyrrhiza uralensis* a 10 to 100 fold increase of **β-radionuclides** in the plant ash of above ground material compared to the soil has been found. For root ash the ratio was with 20 to 160 even higher. For cesium^{137} the ratio was between 2 to 40 for ash from different plants compared to the soil. The use of this and other endemic species from this region is very promissing aspect for phytoremediation of this unique contaminated area where more than 20% of all atomic bombs have exploded on this planet. For many countries in Asia and Europe it is especially important to realize that from unvegetated soils there is a significant transfer of radionuclides via the air into the food chain of many other countries of this planet which could continue over long periods of time if we do nothing.

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CAN AQUATIC PLANTS REMOVE HEAVY METALS FROM INDUSTRIAL WASTES AND SLUDGES EFFECTIVELY?

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1. Introduction

The aquatic fern *Azolla,* a traditional biofertilizer, which forms a symbiosis with the N_2-fixing cyanobacterium *Anabaena Azollae* is distributed worldwide and is employed as green manure by rice farmers. *Azolla* is an exceptionally efficient producer of biomass, due to its high photosynthetic bioproductivity and simple vegetative propagation, and is easily converted to available N-source in flooded rice farms.

We identified the uptake, accumulation and storage of toxic heavy metals by *Azolla* grown in the presence of 1-20 ppm of Cd, Cu, Zn, Cr and Ni ions and described the immobilization of these metals in the cell wall and in the insoluble fraction of *Azolla* biomass (Sela et al., 1989; Tel-Or et al., 1997). Furthermore, we found that the metal binding groups of dried *Azolla* biomass, packed in a column, are active in biosorption of toxic metals when polluted waste pass through the *Azolla* matrix. The metal binding properties of *Azolla* matrix were used to develop the *Azolla* biofilter, on a small laboratory scale, to demonstrate and test its ability to filter larger volumes of industrial wastes (Tel-Or et al., 1991).

2. *Azolla* Metal-binding Groups

We failed to identify or to isolate phytochelatins in *Azolla* grown in mineral medium in the presence or in the absence of Cd^{+2} and Cu^{+2}. Fractionation of water-soluble proteins from *Azolla* yielded three major fractions of metal bound proteins, which were analyzed for their Cd content and affinity to DEAE-cellulose. Further chromatography on Sephadex G and additional separation on 20% native gel-electrophoresis led to the separation of 2 novel 10 kD metal binding proteins. Amino acid sequence analysis demonstrated partial homology between peptides A and B. Peptide A is composed of 15 aspartate, 11 residues of glutamate and 3-4 cystein residues. Its N-terminal sequence contained 20 amino acids – phe-leu-val-thr-leu-leu-tyr-X-asn-asn-X-gly-gln-X-thr-leu-ser-tyr-pro-lys (ala). Peptides A and B contained two identical sequences of 13 amino acids - phe-leu-val-glu-ser-asp-thr-ser-asp-asp-val-phe-lys and 16 amino acids – leu-ala-thr-asp- asp-leu-val-pro-phe-val-leu-gly-thr-thr-glu-ser. However, the sequence of peptide A contained an additional amino acid sequence (17) which did not appear in the Peptide B - gln (or ser or gly)- ile-arg-ser-tyr-val-ala-thr-X-ala-thr-asp-ile-ile-ile-val-asn.

Binding of Zn^{+2} and Cu^{+2} by a purified protein band on gel-electrophoresis was analyzed by X-ray-fluorescence spectrometer. It is suggested that free carboxyl residues of aspartate and glutamate are involved in the binding of Cd^{+2}, Cu^{+2} and Zn^{2+} by this protein, which may function in the translocation of these ions in the shoot of *Azolla* (Nahum-Schneider, 1999).

The primary candidates for heavy metal immobilization in *Azolla* matrix is pectin, consisting of 3-4 percent of the *Azolla* dry weight. Pectinase treated *Azolla* biomass lost a significant part of its

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metal binding capacity, tested for Sr^{+2} binding and, when free carboxyl groups were blocked by methylation, *Azolla* biomass retained only part of its metal binding capacity. Demethylation of this matrix, regenerated the original metal binding capacity of *Azolla.* Thus, pectin moiety in the *Azolla* cell wall plays a major role in heavy metal binding. We have also searched for polyphosphates in the P-rich *Azolla* biomass, known for its P-storage capacity. Fractionation of P-containing residues in acid treated and non-treated samples, analyzed spectrophotometrically and by P-NMR, indicated that a major percentage of phosphate is retained in phytic acid residues. Phytate fractionation followed by equilibrium dialysis experiments, demonstrated Cd^{+2} binding by phytate. Phytate may be stored in the vacuoles of *Azolla* shoots and sequester excessive soluble heavy metal ions.

3. Sludge Treatment by *Nymphaea*

We have introduced a second approach for heavy metal removal from contaminated sludge, aiming to remediate Cd, Hg, Pb, Co and B accumulated at two industrial sites in Israel. The water lily *Nymphaea* demonstrated tolerance to high concentrations of these toxic elements in sludges containing concentrations of 70-450 mg/kg Cd and 1-3.5 mg/kg Hg. Moreover, *Nymphaea* growth cycles in flooded sludge for one month displayed a significant reduction in Cd content - from 70 to 15 mg/kg - in one site and from 450 to 220 mg/kg in another site of polluted sludges. The toxic metal ions are stored in the root and the shoots of *Nymphaea,* and petioles and blades show high affinity to Cd^{2+} ions. Currently we are studying the mobilization of the toxic metals in sludge and their uptake and immobilization by the biomass of *Nymphaea.*

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5. Acknowledgements

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BIOREMEDIATION OF METAL-CONTAMINATED SOILS

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Bioremediation refers to the use of natural or genetically-modified microorganisms (or plants) to clean-up soils contaminated with pollutants. Microorganisms hold great promise as tools to bioremediate contaminated soils due, in part, to their metabolic versatility and their ability to rapidly transform a large variety of compounds. While ideal bioremediation approaches detoxify or mineralize pollutant compounds, and thereby remove contaminants from the environment, in some instances microbial activity merely renders the compound less hazardous than the parent material, transforms the compound to a form that can be chemically/physically separated from soil particles, or sequesters pollutants in soils or sediments in a biologically inactive form (Sadowsky, Turco 1999). Combining microbial metabolism with chemical and physical treatment often represents the most cost effective approach for decontaminating polluted soils (Madsen 1991). However, to use this approach, one must have a complete knowledge of the interaction between the contaminant, microbes, and their local environment (Madsen, 1991; Stroo 1992; Tiedje 1993). While both *ex-situ* and *in situ* bioremediation procedures have proven useful to clean-up contaminated soils, the latter bioremdiation techniques are often advantageous when contaminants occupy a relatively large surface area or the contaminants have moved sufficiently deep into the soil profile to make soil excavation economically infeasible. Many studies have shown that *in situ* biodegradation of contaminated soils can be enhanced by adding limiting nutrients, microbial growth factors, or surfactants to the contaminated environment (Miller 1995; Prince, 1993, Stroo, 1992). The ultimate goal of any bioremediation program is to overcome natural soil heterogeneity, optimize microbial growth conditions by removing rate limiting factors, and allow microbial communities to fully utilize the pollutants (Sadowsky, Turco 1999). It should be noted, however, that sorption and desorption of compounds to soil particles greatly influences their biodegradability by directly influencing bioavailability (Moorman 1993).

Contamination of the environment with metals and metalloids is due to both anthropogenic and natural causes. However, agricultural activity, manufacturing, mining, and the land application of sewage, have contributed a large amount of metals to the environment in a relatively short period of time (Gadd, White 1993). Microorganisms represent one of the major factors affecting the solubility, bioavailability, mobility, and presence of metals in the environment. Several factors have been shown to influence the remediation of metal contaminated soils, including: the ionic form of the metal, electron donors, presence of electron acceptors, soil N and P status, and soil environmental factors (pH, Eh, moisture, organic matter content, and temperature) (Francis 1999). The metals Pb, Ag. Pt, Au, Ga, Cd, Co, Cr, Ni, Fe, Hg, Zn, and the radionuclides ^{60}Co, ^{137}Cs, ^{85}Sr, U, Th, Ra, Am and Pu have been targeted for microbial-mediated bioremediation. Contamination of soils with metals, metalloids, and radionuclides in pervasive, in a five year period beginning in 1982 it was estimated that 50% of U.S. EPA Superfund sites were contaminated with inorganic heavy metals (Frankenberger, Losi, 1995). Generally speaking, biosorption, microbial leaching, precipitation, and direct transformation processes can be used to clean-up soils impacted by metals and metalloids. Microorganisms (bacteria, yeast, cyanobacteria, and filamentous fungi) play a significant role in transforming and accumulating metals in soils. Biosorption, using whole-living or dead biomass, either encapsulated, free, or immobilized on particles and support matrices, has proven to be very effective at removing metal contaminants from a variety of environments (Gadd,

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White 1993). Microorganisms can also directly solubilize metals in soils by oxidation, reduction, methylation, and dealkylation reactions. In some cases the metal is used as a terminal electron acceptor and in others, enzymatic, reductive-dissolution of metals by facultative and anaerobic microorganisms transform metals to a more soluble form. Metals in soils may also be reduced and precipitated by gaseous bioreduction reactions, usually by the action of H_2S produced by sulfate-reducing bacteria and crystallize metals in a mineral form (Francis 1999). Metal binding proteins and peptides (metallothioneins and □-glutamyl peptides), microbial cell wall components and exopolymers, have been shown to specifically remove metals from soils and soil solutions. Lastly, rhizospheric microorganisms, working in conjunction with hyperaccumulating plant species offer great future promise as a cost-effective and visually pleasing means to decontaminate metal-polluted soils (Sadowsky, Smith 1996).

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HOST-PLANT FACTORS IN THE ADAPTATION OF INDIGENOUS AFRICAN LEGUMES TO LOW pH SOILS

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1. Introduction

The pH of soils in the western Cape varies considerably from pH 3 in acidic sands up to pH 8 (or more) in a few isolated calcareous soils. Nutrient levels in these acidic soils are extremely low to support growth of most crop plants, especially symbiotic legumes. However both legume and non-legume species indigenous to these environments grow well despite the prevalence of acidity and nutrient stress. Because the south western Cape is one of the most species-rich plant kingdoms in the world, many legume and non-legume plants occur here that are not found anywhere else in the world. This suggests the possible presence of unique biological traits or specialized adaptations, be they physiological, biochemical, morphological or anatomical, that enable these plants to cope with acidity and nutrient stress in their environments. Nitrogen-fixing legumes are of particular interest because of the sensitivity of the symbiotic process to acidity and low nutrient concentrations. The aim of this study was to determine how indigenous legumes cope with pH and nutrient stress during plant development.

2. Materials and Methods

Field studies were conducted on species of *Cyclopia, Aspalathus, Lupinus* and other unidentified legumes indigenous to the western Cape. Legumes growing in soils of different fertilities and at different elevations (from sea level up to 5 000 m) were selected. Samples of plants and soils were taken and analyzed for nutrients, pH, symbiotic dependence through ^{15}N natural abundance measurements of leaf tissues, modification of rhizosphere pH, and observation for adaptive characters such as cluster roots.

3. Results and Discussion

Field measurements of symbiotic functioning in *Aspalathus linearis, A. carnosa, Cyclopia intermedia, C. maculata, C. sessiliflora, C. meyeriana, C. subternata, C. genistoides,* and *Lupinus* spp. showed high levels of nodulation and N fixed in nutrient-poor acidic soils of the western Cape. However the $\delta^{15}N$ values obtained, which ranged from 0 ‰ to - 1.0‰, differed markedly between genotypes, species and location. These differences in nodule function or $\delta^{15}N$ values were related to variations in soil pH, nutrient levels and soil moisture content. The fact that *rhizobium* nodulation, which enhances N nutrition of legumes, was not hampered in nutrient-limited acidic soils, suggests that these plants must have mechanisms for promoting nutrient acquisition. As shown in Table 1, *Cyclopia genistoides* can actively modify its rhizosphere pH for increased nutrient availability to plant roots. Similar data have been obtained for *A. linearis* and other species within this genus which grow in soils at pH 3-4 and yet fix large amounts of N (Muofhe and Dakora 1999). These legumes not only increase the pH of their rhizospheres for symbiotic establishment but also for enhanced nutrient availability. Titrimetric studies show that the elevation in rhizosphere pH is caused by extrusion of OH^- and HCO_3^- by roots of *A. linearis,* and is probably the case with *C. genistoides.* Alkalinization of the rhizosphere from exudation of OH^- and HCO_3^- is, in evolutionary terms, an important trait in indigenous legumes adapted to growth in acidic soils.

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Table 1. Chemical modification in rhizosphere soil vs. non-rhizosphere bulk soil (0-15 cm) of *Cyclopia genistoides* growing at sea level (Rondeberg) and at 1000 m elevation (Constantiaberg)

Chemical parameter	Rondeberg rhizo.	Rondeberg non-rhizo.	Constantiaberg rhizo.	Constantiaberg non-rhizo.
pH (KCl)	5.0 ± 0.2	4.4 ± 0.1	4.0 ± 0.3	3.6 ± 0.5
C (%)	1.1 ± 0.3	0.7 ± 0.1	7.6 ± 0.6	3.9 ± 0.8
P (mg/kg)	4.8 ± 0.9	3.3 ± 0.9	11.0 ± 1.4	5.0 ± 1.0
K (mg/kg)	33.0 ± 11.3	15.8 ± 3.3	97.3 ± 9.6	37.3 ± 9.6
Ca (me%)	3.3 ± 1.0	1.5 ± 0.4	5.2 ± 1.9	2.1 ± 0.3
Mg (me%)	1.3 ± 0.4	0.4 ± 0.1	2.5 ± 0.2	1.1 ± 0.1
B (mg/kg)	0.7 ± 0.3	0.2 ± 0.02	1.3 ± 0.3	0.6 ± 0.2

Rhizo = rhizosphere soil; non-rhizo = non-rhizosphere soil. Mean ± SD

Because the soils supporting growth of these legumes are very acidic, they are also low in P concentrations. Field survey of test legumes have revealed the presence of massive cluster roots (proteiod roots) in field plants of*Aspalathus linearis, A. carnosa, Lupinus* and *Cyclopia* spp., many unidentified legumes. In lupins, the specialized clusters of closely packed lateral rootlets exude organic molecules, namely citric, malic and succinic acids (Johnson et al. 1996; Neumann et al. 1999), which solubilize P and oxides of Fe from otherwise insoluble forms for uptake by the plant. It is therefore likely that cluster roots of *Aspalathus, Cyclopia* and the unidentified legumes also exude these organic acids to optimize P nutrition in the P-deficient acidic soils of the Cape. Although the C cost of cluster root formation and organic acid exudation could constitute a drain on the plant's total photosynthate supply, fortunately 25% of the C for citrate and 34% of C for malate exudation come from nonphotosynthetic CO_2 fixation by phosphoenolpyruvate carboxylase in roots (Johnson et al. 1996). In addition to organic acid exudation, the rhizoplane of proteoid roots is characterized by different pH regimes which facilitate solubilization of P in low pH soils, with the mature proteoid part tending to be lower in pH compared to the non-proteoid apical zone or the juvenile proteoid parts (Neumann et al. 1999). These indigenous legumes also have an affinity for root infection by fungal symbionts. Species of *Cyclopia, Aspalathus,* and other unidentified legumes have heavy root infections of VA mychorrizae for enhancing P nutrition and water uptake in the dry low-nutrient soils of the Cape. Clearly, the legumes and microbes of these acidic ecosystems provide both a challenge and an opportunity for understanding plant response to stress, knowledge of which could boost crop yields in these nutrient-poor acidic environments.

4. Acknowledgement

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A NEW REMEDIATION SYSTEM FOR HEAVY METALS USING LEGUMINOUS PLANT AND RHIZOBIA SYMBIOSIS

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1. Introduction

Industrialization has brought many unexpected problems on our earth. In Japan, peoples have suffered from ill-defined syndromes, such as Itai-itai disease or Minamata disease. Now, we know that these diseases resulted from foods, which contained a large amount of Cd and Hg, respectively. Usually, we have a protection mechanism in our body against these toxic heavy metals. Metallothioneins (MTs) are low-molecular weight, cysteine-rich proteins that bind heavy metals such as Cd, Zn, Hg, Cu, Ag, etc. Mammalian MT-II consists of 61 amino acid residues that include 20 cystein residues that bind 7 gram atom metals per one molecule. However, MTs are unstable when expressed in bacteria (Murooka et al. 1987). To stabilize MTs in bacteria and to increase the binding of heavy metals, we designed oligomeric MTs. To develop remediation system for heavy metals in polluted field, we used renge-sou (*Astragalus sinicus*), which has been used as a green manure in Japan and China more than 1000 years.

2. Results and Discussion

We developed a new remediation system for heavy metals using symbiosis between leguminous plant, renge-sou (*Astragalus sinicus*) and rhizobia, *Mesorhizobium huakuii* subsp. *rengei,* which was isolated from nodules on *A. sinicus* cv. Japan (Murooka et al. 1993, Nuswantara et al. 1999). To increase the metal binding capacity, we constructed oligomeric metallothioneins (MTs) and used these constructs for absorption of heavy metals. The designed hMT-II DNA oligonucleotides were synthesized as described previously (Yamashita et al. 1994). The dimeric and tetrameric MTs were overproduced in bacteria and bound two and four times Cd and Zn, respectively, than the hMT monomer. Transformation systems both in *M. huakuii* and renge-sou plant were developed (Cho et al. 1998). *M. huakuii* subsp. *rengei,* which contains the oligomeric MT gene, was infected and formed nodules on renge-sou plant.

Renge-sou plant was planted in planter containing 20 to 500 ppm Zn, Cu, or Cd in the soil. After growing the plant, heavy metal contents in shoots and roots of plants were analyzed. Zn was accumulated in the plant 10 times more than Cd. The transportation from roots to shoots depended on heavy metals, in case of Cd 10 % of total contents was translocated to shoots, whereas 50 % of Zn was translocated from roots to shoots. The transgenic renge-sou plant containing the tetrameric MT gene expects to removal 20 times heavy metals (about 250 grams of Cd / 10a) than the wild-type plant from polluted soil.

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UV -B EFFECTS ON PLANT GROWTH, SYMBIOTIC PERFORMANCE AND METABOLITE CONCENTRATION IN TROPICAL LEGUMES

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1. Introduction

UV-B radiation has adverse effects on growth and metabolism of many plant species (Caldwell 1979) due to the high absorption affinity of macromolecules such as proteins and nucleic acids in that wavelength. As a defence mechanism, plants accumulate pigments such as flavonoids and anthocyanins in response to UV-B radiation (Teramura 1983). However flavonoids and anthocyanidins from seeds and roots of nodulating legumes serve as transcriptional regulatory signals to symbiotic bacteria in the Rhizobiaceae (Hungria et al 1991, Phillips 1992). The accumulation of these pigments in plant organs such as seeds and roots could therefore influence nodulation in N_2 fixing legumes. The aim of this study was to assess the symbiotic performance of plants exposed to UV-B and its effects on the F1 generation plants. Data on the former are reported here.

2. Materials and Methods

Four tropical legumes namely cowpea, soybean, bambara groundnut and common bean were grown outdoors in pots under ambient and elevated UV-B at Kistenbosch Botanical Institute, Cape Town. Elevated UV-B was supplied by artificial UV-B supplementation with filtered UV fluorescent sun lumps. Weighted doses in the ambient UV-B treatment was 3.66 kj m^{-2} d^{-1} (average amounts for the experimental time covering February to June in Cape Town) and elevated UV-B was 8.09 kj m^{-2} d^{-1}. The elevated UV-B amount simulated those that would be received in the tropical region, southern latitudes. All the plant species were assessed for nodulation, nitrogen fixation, biomass accumulation, and concentration of metabolites at 65 and 86 days after planting (DAP)

3. Results and Discussion

Nodule mass was unaltered in all the species exposed to elevated UV-B except cowpea which had a significant ($P<0.05$) 15 % reduction. Total amount of nitrogen fixed per plant, concentration of polyphenolics and total biomass were also not affected in all the plant species. Root mass was however significantly reduced in all species except common bean. Although the concentration of carotenoids was markedly reduced in soybean, the levels of soluble sugars , starch and total non-structural carbohydrates (NSC) increased. Chrolophyll a/b ratio and total NSC also increased significantly ($P<0.05$) in cowpea and bambara groundnut respectively. Although nodulation was affected in cowpea, total nitrogen fixation was not affected in all the species. The lack of significant differences between UV-B treated and ambient plants in nodulation and nitrogen fixation was not surprising because our elevated UV-B radiation (7.13 kj m^{-2} d^{-1}) treatment was about the same amount of UV-B radiation experienced by the species in the tropics.

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ULTRASTRUCTURE OF COIMMOBILIZATION OF THE MICROALGA *CHLORELLA VULGARIS* WITH THE PLANT GROWTH-PROMOTING BACTERIUM *AZOSPIRILLUM BRASILENSE* AND WITH ITS NATURAL ASSOCIATIVE BACTERIUM *PHYLLOBACTERIUM MYRSINACEARUM IN* ALGINATE BEADS

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The wastewater treatment microalga *Chlorella vulgaris* was coimmobilized and coincubated with the plant growth-promoting bacterium *Azospirillum brasilense* or with its natural associative bacterium *Phyllobacterium myrsinacearum* in alginate beads designed for wastewater treatment. Most small cavities formed within the bead were colonized as microcolonies by one microorganism regardless of the bacterial species accompanying the microalga. Later, the bacterial and microalga microcolonies "fused" to produced a joined, larger, mixed colony in the cavity. Then, the events within the cavity differed depending on the bacterial associate in the coimmobilization. Though the microalgae became senescent in the presence of *P. myrsinacearum,* it remained juvenile and continued to multiply in the presence of *A. brasilense*. This study proposes that there are possible commensal interactions between the microalga and the two plant associative bacteria.

MICRONUTRIENT REQUIREMENT FOR THE GROWTH SURVIVAL AND SYMBIOTIC ASSOCIATION OF SOME RHIZOBIAL STRAINS

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Molybdenium, iron and zinc requirement for growth and survival of fifteen strains of rhizobia representing *Bradyrhizobium japonicum, R. leguminosarum bv. viceae* and *phaseoli* was tested in culture and carrier media. Siderophore production of the tested strains was investigated, as well as the symbiotic performance of the legume-*Rhizobium* association as affected by the micronutrient application and/or mycorrhizal inoculation in newly reclaimed soil. The result shows that, both growth rate and maximum cell yield of rhizobia varies greatly between species and among strains in response to different levels of micronutrient in a chemically defined media. A positive correlation between rhizobial cells (number and survival) and the combined micronutrients, at their optimum levels was observed. This effect was more pronounced in vermuculite than in peat. Most strains *of R. leguminosarum bv. viceae* and *phaseoli* exhibited a remarkable response to siderophore production. The local strain ARC206 of *R. leguminosarum* was the best siderophore producer. On a newly reclaimed sandy soil, the soybean plants treated with micronutrients and inoculated with both rizobia and mycorrhiza recorded the highest values of growth and yield, as well as nutrient content of seeds.

SECTION XIII

STRAIN SELECTION AND INOCULATION TECHNOLOGY

LEGUME INOCULATION IN CERRADO SOILS

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The Brazilian cerrado is an edaphic type of savanna that comprises 23% of the country's territory (204 million hectares), characterized by flat land and deep acidic and infertile soils, with high levels of exchangeable aluminum and low cation exchange and water retention capacity. With the development of economically viable fertilization practices, one fourth of this area was incorporated into intensive agriculture systems and today contributes to almost half of soybean and more than one fifth of maize, rice and common bean production in the country.

Cerrado soils have a native rhizobia population that forms an effective symbiosis with some legume species like *Stylosanthes* spp, cowpea (*Vigna unguiculata*), siratro (*Macroptilium atropurpureum*), zornia (*Zornia* spp), calopogonium (*Calopogonium mucunoides*) and lab-lab (*Lablab purpureus*), which grow well in the absence of inoculation and N fertilizer. Other species like *Arachis* spp, mucuna (*Mucuna* spp), pigeon pea (*Cajanus cajan*), crotalaria (*Crotalaria* spp) and lupinus (*Lupinus* spp) are able to fix N_2 with the soil population, but nodulation is poor in the first year and good response is obtained with inoculation or N fertilizer. In contrast, the soil native rhizobia is unable to form nodules with soybean (*Glycine max*), leucaena (*Leucaena* spp.), pea (*Pisum sativum*), lentil (*Lens esculenta*), chickpea (*Cicer arietinum),* guar (*Cyamopsis tetragonolaba*), and alfafa (*Medicago sativa*), and inoculation is mandatory in the first year of cultivation. Common bean (*Phaseolus vulgaris*) nodulates well with native rhizobia in cultivated soils, but most of the nodules are ineffective and interfere with nodule formation with the inoculated strains.

In early seventies, when soybean started to be cultivated in the cerrado, inoculation was not successful due to use of the soybean variety IAC-2, that did not establish an efficient symbiosis with the commercial strains, as well as to the use of low levels of peat inoculants. With the identification of two strains with low host specifity [29W (=SEMIA 5019) and SEMIA 587], the use of higher levels of inoculant (600 to 800 g of inoculant 40 kg^{-1} of seeds) and a 25% sucrose solution for preparing the peat slurry, soybean started to be cultivated in the cerrado without the use of nitrogen fertilizer.

Cultivation of the soils promoted the establishment of adapted rhizobial populations, becoming an important reservoir of genetic material in a selection program. One of those isolates, CPAC15 (serogroup 566), consistently increased soybean yields in field trials and began to be used in commercial inoculants in 1992 (Vargas et al., 1992). Another approach for obtaining efficient rhizobia strains consists in the searching for natural variability within a very efficient but low competitive strain, like CB 1809, brought from Australia to Brazil in 1966. Individual colonies from a pure culture were tested in Leonard jars for competitiveness, and a promising isolate was identified, the CPAC 7. With more nodules occupied by this efficient strain, the rates of N_2 fixation increased and so did the yield in several field trials. Consequently, the strain also started to be commercially recommended since 1992 (Vargas et al., 1992). A positive and important remark in this traditional breeding method is that the stability of the strain is very high. Inoculation with strains CPAC 7 and CPAC 15 promoted yield increases averaging almost 4% above uninoculated plants in bradyrhizobia populated soils, with yields reaching values above 3,500 kg ha^{-1} without N fertilizer. In those soils, the size of the established soil bradyrhizobial

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population interferes with nodule formation with the inoculated strain, particularly when serogroup 566 prevails within the soil population.

Common bean is the most important legume in the Brazilian diet, production centering in small farms where the use of N fertilizer is limited and yields are low, usually below 700 kg ha^{-1}. Inoculation is not adopted by most farmers, even for those using high technologies and accomplishing yields above 3,000 kg ha^{-1}, with heavy use of N fertilizer. Yield response for bean inoculation in the cerrado is variable, ranging from no response to substantial yield increases. Cerrado soils have a low population of bean rhizobia, usually below 10^2 cells g^{-1}, but as the soil is cultivated, the native inefficient population increases in size and prevents nodule formation by the inoculated strains, becoming a major constraint for the success of seed inoculation. In reclaimed flooded soils with no native rhizobia, inoculation promoted over 300% increase in yields, reaching values as high as 2,500 kg ha^{-1} without N fertilizer (Mendes et al., 1994). Cultivation of common bean (with or without inoculation) promotes the increase in the native rhizobia population rather than the inoculated strain, and N fertilizer increases the occurrence in nodules of the native strains. Selection of N tolerant strains from the native population and appropriate N fertilization practices seems to be very promising for reducing N fertilization and still keeping maximum yields.

Isolation of rhizobia strains from soils previously cultivated with peas and lentils was very successful in obtaining very efficient strains for those legumes (Vargas et al., 1994), promoting more than two fold yield increases, reaching values above 3,000 kg ha^{-1} without N fertilizer.

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IMPACT OF NEW TECHNOLOGIES ON INOCULATION BY DIAZOTROPHS

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Introduction

Considerable increases in the human population occurring in recent decades, and the likelihood of continued increases in coming years, have greatly illuminated the now immediate problem of food shortages. Improvements in agricultural products as well as in production practices provide key elements for addressing one component of this immense puzzle.

Land resources available for agriculture are about 20% worldwide, but only half of this land is currently used. Developing strategies for extending agriculture into these unused lands must also employ efficient and sustainable production systems reducing pollution generated by agro-chemicals and nitrogenous fertilizers. Research and development of selected biological resources for soil fertilization may ultimately provide inexpensive, safe and recyclable alternatives.

Optimization of N_2 fixation by plant-diazotrophs associations requires the use of new technologies applicable to both associated organisms. One of these methods involves molecular profiling of strains in order to identify differences in the efficiency and persistence in the soil and in the inoculum. An increasing use of double inoculants has been observed in recent years. In particular, it has been reported the production of inoculants of diazotrophs incorporated with phosphate solubilizing organisms and biological control agents.

In this contribution, a brief review on the latest techniques used for diazotrophs strains selection and characterization and on the improvement of their inoculation is presented.

Molecular profile

The identification of bacteria, as well as the evaluation of their evolutionary genetic relationships has undergone dramatic changes in recent years. Bacterial taxonomy was once restricted to such parameters as colony morphology characteristics, bacteriophage resistance, the presence of some enzymatic activity and nutritional requirements for growth. Although these parameters provide useful information, enhanced resolution has been obtained using methods such as total protein profile analysis, a variety of immunochemical tests, isozyme analysis and in particular total fatty acid analysis.

Technological developments that allow one to determine aspects of the physical structure of the DNA of a given organism have provided the most informative data for the identification of that organism. Such DNA analysis (RAPD's, rep-PCR, RFLP, ARDRA, rRNA sequences), in conjunction with the methods that evaluate the phenotypic properties of an organism, have enabled taxonomist and evolutionary biologist to incorporate an entirely new level of rigor in their studies. As an example, we succeeded in characterizing 465 *S. meliloti* isolates selecting the more effective acid tolerant strain using some of these methods (Del Papa et al. 1999, Segundo et al. 1999).

Plant growth and phosphate uptake. Co-inoculation with phosphate-solubilizing organisms

Most of agricultural soils contain large reserves of phosphorous, a considerable part of which is accumulated as consequence of regular applications of phosphate fertilizer. Evidence indicates that the availability of soil phosphate is frequently a limiting nutrient in alfalfa production. The uptake by plants of fertilizer and soil phosphate is limited by precipitation of phosphate solution with soil cations and by adsorption to soil particles. In fact, a large proportion of soil phosphate is not readily available for plant uptake.

However, there exist a number of microorganisms able to solubilize insoluble phosphate compounds, but this ability is variable among the strains. *Enterobacter sp, Rhizobium leguminosarum* bv

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phaseoli, Penicillium bilaii were found to be very active phosphate-solubilizing organisms (PSO). In this connection, *Enterobacter* sp was selected (Toro et al, 1998) to run an alfalfa pot experiment where it was evaluated the effect of a co-inoculated system formed by *Enterobacter* sp. and *Sinorhizobium meliloti.* This system seemed to improve both the shoot dry weight, phosphate and N uptake by alfalfa. Similarly, the response of alfalfa to dual inoculation with *P. bilaii* and *S. meliloti* was very significant when cultivated in plots during two years (Beckie et al, 1998).

Plant preservation and Biological control

The prevailing trends in agriculture toward greater sustainability and the problem of the use of synthetic pesticides have increased the tendency to introduce microorganisms for biological control of plant diseases. Recent technological advances obtained through genetic and molecular research are enlightening our knowledge on the mechanisms involved in biocontrol agents.

A number of microorganisms have been isolated which are able to suppress plant diseases. The mechanisms involved in this interaction are not well known and especial emphasis is being done to: 1) competition for nutrients for example, carbon and iron; 2) induced resistance; and 3) disease suppression through the production of antibiotics. However, the main interest is given to the detection and characterization of specific antibiotic produced by these biocontrol agents

Several papers have reported: 1) the characterization of phytopatogens agents; 2) the isolation of soilborne microorganisms able to suppress diseases produced by phytopathogen agents. These investigations initially used wheat, maize and tomatoes and, in the last years, legumes such as peas (Xi et al, 1996), lotus (Bagnasco et al, 1998) and beans (Estevez et al, 1999). The already obtained findings allowed the co-inoculation of diazotrophs and the supressor microorganism, increasing substantially the yield crops because of the reduction of the disease effect.

Concluding Remarks and Perspectives

Two main research topics on the inoculation by diazotrophs developed by different laboratories were briefly discussed in this short overview. First, the improvement of molecular techniques based on nucleic acids and microorganism genetic manipulation produced an important impact in the characterization and selection of strains. Secondly, the coinoculation to increase P uptake and the prevention of plant diseases through biocontrol agents, also play a key role in diazotrophic inoculation becoming of considerable importance in agriculture. These and other methods that were not discussed, are powerful research resources with great potentiality to increase the efficiency of soil inoculation.

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ANTIBIOSIS AS A MEANS TO ENHANCE NODULATION COMPETITIVENESS: BEYOND THE MODEL SYSTEM

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At the last International Nitrogen Fixation Congress, we presented the construction of a new plasmid, pHUTFXPAR, that possesses: 1) the trifolitoxin (TFX) production and resistance genes (*tfxABCDEFG*) for improved nodule occupancy; 2) the full complement of genes necessary for uptake hydrogenase for improved plant productivity; and 3) a par locus (*parABCDE*) to ensure plasmid stability in the absence of selection pressure. This plasmid was constructed to improve the ability of inoculant strains to compete for nodule occupancy on legume hosts in agricultural fields containing indigenous rhizobia. Here we present the efficacy of this stable plasmid under field conditions and the use of site-directed mutagenesis for further analysis of the trifolitoxin peptide.

After their transfer into inoculum strains of *R. etli* and *Sinorhizobium meliloti,* seeds of snap bean and alfalfa, respectively, were coated with pHUTFXPAR. Additional treatments included an uninoculated control and various control plasmids described previously (Kent et al 1998). The presence of *tfxABCDEFG* and *parABCDE* in an inoculum strain were required for increased nodule occupancy in both alfalfa and bean against indigenous rhizobia and/or a marked, reference co-inoculum strain. Alfalfa plots inoculated with a strain of *S. meliloti* containing pHUTFXPAR significantly increased forage yield compared to either the uninoculated or wild-type inoculum strain controls. In greenhouse and field experiments with *Phaseolus vulgaris,* nodule mass and nodule occupancy significantly increased in the pHUTFXPAR treatment compared to the above control. The variability of the field experiment was too high to assess yield response on bean. To date, no plasmid transfer between the inoculum strains and indigenous rhizobia has been observed.

We have now shown that the TFX production phenotype has significantly increased nodule occupancy by the inoculum strain on *Phaseolus vulgaris* over four field seasons and at three sites using three different plasmid constructs that confer TFX production. Some of this work has been published (Robleto et al, 1998a). For *Medicago sativa,* we now have evidence from two sites over two years with two different plasmid constructs showing increased nodule occupancy in the field. The increases obtained are in the range of 20-40% over the wild-type strain. Thus, we are confident that the TFX production phenotype can increase nodule occupancy of inoculum strains for many legumes under a variety of field conditions. The potential yield benefits of the uptake hydrogenase phenotype in inoculum strains requires more study.

We are also examing approaches to further understand and improve the TFX system. Site-directed mutagenesis is being used to: 1) determine those amino acids that are essential to the TFX peptide; 2) improve the system by identifying a mutation with increased activity; 3) determine whether *tfxA* expression is limiting to TFX production; and 4) construct structurally simpler, active peptides that can be used in the final determination of the TFX structure. The assay system for the site directed mutagenesis includes the construction of a strain that contains all of the genes for TFX production and resistance on the chromosome except for an in-frame deletion of codons 37 and 38 from the *tfxA* gene. We refer to this mutant as **T24::Tn5_4A112**. This allows the addition of *tfxA in trans* on a broad-host-range plasmid, pRAWT, to complement the TFX production phenotype. Mutated *tfxA* genes are also added to **T24::Tn5_4A112** in this manner to test the phenotype of specific mutations.

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With this assay system, we are able to determine whether *tfxA* expression is limiting in TFX production. Preliminary results suggest that with multiple copies of *tfxA* provided on a plasmid, significantly more TFX was produced by **T24::Tn5$_4$A112(pRAWT)** than by the wild-type strain. The wild-type strain, **T24::Tn5$_4$,** possesses a single copy of *tfxA* on the chromosome. The significance of this result is that we should be able to confer much more chromosomal TFX production than is now available simply by increasing the strength of the *tfxA* promoter. This would permit the construction of very competitive strains without the need for the addition of a stable plasmid but rather with the addition of a *tfxABCDEFG* cassette containing the stronger *tfxA* promoter.

To date, mutations in *tfxA* codons 36-39 have been made. These codons code for three of the four amino acids that are post-translationally modified as well as a serine upstream of these modifications. Mutations in the 37-39th codons resulted in complete loss of TFX activity. These mutations include R37G, Q38A, Q38E, and G39A. Given the model for the structure of TFX, these amino acid residues are all post-translationally modified to create the active peptide. Previous results showed that the chemically synthesized, unmodified peptide with the amino acid sequence, DIGGSRQGCVA, had no activity and that the cyclization of cysteine was not required for TFX activity. The above site directed mutations give us the first direct evidence for the role of these residues in TFX activity. Alanine scanning of the entire peptide is now in progress to determine the role of each amino acid in TFX activity.

The 36th codon has been mutated from serine to alanine (S36A), threonine (S36T), and tyrosine (S36Y). A strain carrying the S36T mutation was unaltered in TFX activity while the S36Y-containing strain was significantly reduced in antibiotic production. The S36A mutation conferred slightly increased antibiotic activity compared to **T24::Tn5$_4$A112(pRAWT)** against one test strain. This result suggests that additional mutagenesis of *tfxA* may result in peptides with significantly more activity than TFX. Experiments are in progress to test the hypothesis that *tfxA* is monocistronic.

In summary, we have shown that the TFX production phenotype enhances the nodulation competitiveness of inoculum strains that infect *Phaseolus vulgaris* or *Medicago sativa* in the field. These results are not surprising given the dramatic effects that a TFX-producing strain can have on the diversity of TFX-sensitive bacteria in the rhizosphere (Robleto et al, 1998b). We have preliminary evidence that the addition of the uptake hydrogenase phenotype along with the TFX production phenotype may enhance legume yield as well under agricultural conditions. Genetic efforts are in progress to further improve the TFX system through site directed mutagenesis.

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HORIZONTAL GENE TRANSFER IN RHIZOBIA: ECOLOGICAL IMPLICATIONS

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1. Introduction

Evidence collected over the last years indicate that exchange of symbiotic information among rhizobia may be a common event. Conjugations seems to be the prevalent mechanism for transfer of plasmid-borne as well as chromosomal genes from symbiotic rhizobia to other symbiotic and non-symbiotic bacteria. Although transfer can be shown in the lab, it is extremely difficult to quantify in the open field. However, there are indications that transfer occurs in soil and that this phenomenon may be relevant for evolution of rhizobia and could have important ecological consequences.

2. Horizontal gene exchange in rhizobia

We have previously reported that the *R. meliloti* genome contains multiple mobilizable (Mob) regions which represent high-frequency conjugative transfer systems. Some large replicons contain several Mob sites, indicating that transfer occurs separately for different replicon segments (Herrera-Cervera et al., 1998). Transfer frequencies of such Mob DNAs depend on the genetic background of the donor and recipient cells, as well as the environmental conditions. Transfer of cloned *oriTs* from several *R. meliloti* strains to a particular *R. meliloti* recipient strain was found to be dependent on the plasmid content of the donor. Likewise, transfer of Mob DNAs from a particular donor greatly varied upon the recipient bacterial species. Moreover, transfer efficiency to soil isolates belonging to the same 16S species differ by several orders of magnitude, indicating that not all potential recipients have the same opportunities for DNA exchange under a particular situation. In addition, transfer of these Mob regions is severely reduced in media with ammonia as the only nitrogen source. This resembles a previous report on ammonia inhibition of plasmid transfer in *R. meliloti,* showing that in the presence of ammonia, potential recipients were reluctant to acquire foreign DNA (Herrera-Cervera et al. 1996). It is also intriguing that ammonium, the ultimate product of nitrogen fixation, may be limiting DNA exchange between nitrogen-fixing bacteria.

The data presented above focus on the mechanistics of DNA exchange and indicate that virtually any part of the rhizobial genome can be subject of exchange. Studying them will provide essential information as to what and when genetic information can horizontally spread. However, for DNA exchange become ecologically significant, the DNA transferred must be stably maintained in the recipient (either by self-replication or by recombination) and should provide useful information to the recipient in order to gain some adaptive advantage in the soil. In the case of exchange of symbiotic information, the transconjugants may become direct competitors of the donor for nodulation of one or more legume species, especially when the donors are introduced into a new site and the recipients are native bacteria, well adapted to the general soil conditions. Competition, thus, may lead to a total or partial replacement of the introduced strains by newly emerging strains. The time scale over which process takes place depends on many factors, mainly the relative saprophytic and nodulating competence of the strains involved, but also on the type and frequency of legume and non-legume crops. Two examples of replacement of introduced strains after donating symbiotic information to indigenous bacteria are reported, in which replacement time greatly vary from years to centuries.

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Example 1: Horizontal spread of biovar phaseoli symbiotic plasmids.
The origin of *Phaseolus vulgaris* is believed to have been Meso-America. This crop was introduced in Europe after XVI century, and probably its rhizobial symbionts were also introduced as seed contaminants. At the sites of origin, two symbiotic rhizobial species are predominant, *R. tropici* and *R. etli,* the latter representing bv. *phaseoli,* characterized by carrying multiple copies of the *nifH* gene on the symbiotic plasmid. *R. etli* strains have been found in European soils. However, bv. *phaseoli* strains carrying similar if not identical sym gene organization, belong to at least three additional species, *R. leguminosarum, R. gallicum* and *R. giardinii.* Thus, it seems that after introduction of *R. etli* as seed contaminants, these bacteria have donated their symbiotic plasmids to other species. Currently, three type situations can be described in European soils with regard to bv. *phaseoli* strains:

a) bv. *phaseoli* is represented exclusively by *R. etli* strains (Sessitch et al. 1997): in these soils, no significant pSym transfer has occurred.
b) *R. etli* strains coexist with strains carrying virtually identical bv. *phaseoli* pSym, but belonging to different species: the extreme case is that of a Spanish soil where up to four species carrying identical *nifH* gene organization coexist (Herrera-Cervera et al. 1999).
c) no *R. etli* strains can be found, however there are bv. *phaseoli* strains of other species (Geniaux et al. 1993; Amarger et al. 1997). These could represent soils where introduced *R. etli* strains have been replaced by strains arisen after acquisition of bv. *phaseoli* pSym. In this case, however, other explanations cannot be ruled out.

Example 2: Transfer of a M. loti chromosomal symbiotic island (Sullivan, Ronson, 1998).
This case represented the ideal situation for introduction of a rhizobial inoculant: a nitrogen-poor soil, devoid of nodulating rhizobia, where a new crop, *Lotus corniculatus,* is introduced along with an inoculant strain of *M. loti.* Seven years after introduction, only 19% of nodules are occupied by the inoculant strain, and most nodules are infected by genetically diverse strains that acquired symbiotic genes from the inoculant strain. Thus, is a typical case of introduction of a new rhizobia and its replacement by transconjugant bacteria that are highly competitive and better adapted to the soil. The replacement time is unusually short and probably represents an extreme, but nevertheless, real case of loss of inoculation efficacy due to horizontal gene transfer.

3. Conclusions

The direct consequences of this phenomenon are loss of inoculation efficacy, as the introduced inoculant strain can be outcompeted by new emerging strains, which could be poor nitrogen-fixers but will occupy most of the nodules, providing little nitrogen to the current crop. Similar problems may arise if new legume genotypes are introduced, along with new inoculants, in the future.

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PLANT-BIOTIN A POSSIBLE SIGNATURE COMPOUND FOR *SINORHIZOBIUM MELILOTI* DURING ALFALFA ROOT COLONIZATION

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Plants release a wide variety of chemical compounds, which are then available to affect microorganisms in the surrounding soil. Only recently we have shown that plant-derived biotin is an important factor stimulating *S. meliloti* growth in alfalfa rhizospheres (Streit et al 1996). Already nM amounts of biotin increased *S. meliloti* colonization of alfalfa roots. However, the molecular mechanism by which external biotin from the alfalfa plant stimulates growth of *S. meliloti* cells is unclear. Early work by West and Wilson and more recent work by Sierra et al. showed that *S. meliloti* requires no biotin for growth but produces small amounts of biotin. Work in our group tries to identify possible molecular mechanisms *S. meliloti* might employ to benefit from plant-exuded biotin. We have identified two DNA-loci regulated by biotin and not linked to biotin synthesis: (I) BioS, a possible LysR-type regulator and (II) a polyhydroybutrate (PHB) dehydrogenase gene involved in PHB mobilization.

(I) One possible mechanism involves *bioS,* a biotin-inducible gene in *S. meliloti* that affects competitive growth (Sierra et al 1999, Streit, Phillips 1997). Because *bioS* is induced in response to exogenous biotin when the cells have been transferred through several passages of biotin-free media, we speculate that BioS protein is involved in sensing plant exuded biotin in a rhizosphere. Interestingly our data indicate that BioS protein probably contains three helix-turn-helix motifs found in other transcriptional activator proteins such as the *S. meliloti* NodD and SyrM proteins or in the LysR protein of *E. coli.* BioS, as a small receptor protein, could be involved in the process that helps *S. meliloti* to make the transition from a dormant, resting soil microbe to a fast-growing, competitive root colonizer. New data supporting the relevance of this gene to plant-microbe interactions show that a *bioS-gusA* reporter fusion is expressed by bacteria on plant roots, by bacteria in alfalfa root nodules, and more generally by any stationary-phase bacterial cells in the presence of biotin. Significant transcription of *bioS-gusA* was detected at 50 pM biotin, and an overall 16-fold induction was measured in stationary-phase cells with 1 nM biotin. A positive autoregulation of *bioS* is suggested by the fact that in the presence of 10 nM biotin, a *bioS-gusA* fusion in wild-type cells was transcribed at more than twice the rate of the same reporter fusion in a *bioS* mutant background. Analyses of primer extension products and expression studies using three different *bioS-gusA* promoter fusions defined major characteristics of the *bioS* promoter including three $\mathbf{T\text{-}N_{(11)}\text{-}A}$ motifs upstream from the *bioS* gene. A regulatory role for BioS protein was supported by tests showing that a 321-bp DNA fragment containing the *bioS* promoter altered the electrophoretic mobility of recombinant BioS and of a corresponding band in protein extracts from *S. meliloti* 1021.

(II) Another mechanism *S. meliloti* might employ to benefit from plant exuded biotin could involve the *bdhA* gene linked to PHB mobilization in S. *meliloti* (Aneja, Charles 1999). A rapid mobilization of stored PHB in response to plant exuded biotin would allow the bacterium to rapidly colonize the plant rhizosphere and thus contribute immediately to root nodule formation and N_2 fixation. When cells of *S. meliloti* were grown under biotin limiting conditions and analyzed by electron microscopy, we observed a high contents of PHB in these

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cells. In contrast cells which had been grown in the presence of biotin did not contain stored PHB granules. Because under the biotin-limiting conditions the *S. meliloti* cell lumen was filled with PHB granules but not in the presence of of biotin we hypothesized that either PHB synthesis or degradation are significantly influenced by biotin present in the culture medium. To test this hypothesis the *S. meliloti* 3-hydroxybutyrate dehydrogenase gene (*bdhA*) was mobilized as a *bdhA-lacZ* fusion into the *S. meliloti* 1021 wild-type strain, **β-galactosidase** tests revealed an overall 4 to 5-fold higher *bdhA* transcription in the presence of added biotin. An alignment of the *bdhA* and the *bioS* promoter regions identified several common motifs possibly forming a *bio box* in *S. meliloti* biotin regulated genes (Figure 1).

```
                                  -35                  -10
bdhA: TTGCCGGGCGGAAAAACACAATTGTCACTGCCGCGCATTTGCGCGTAGGCTGACGCTGT
bioS: CAGGCCGGGACAGCATGAACATGGCGCGGAGTGTGCATTTGCTCCCCATCCCGGTCGTCGT

possible bio box motifs:
      --GNCNGG------------ATNG--------GNGCATTTGCNC
```

Figure 1
Comparison of the *S. meliloti bdhA* and *bioS* promoter region. Identical nucleotides in the promoters as part of a possible *bio* box are underlined. Transcriptional start codons as determined by primer extension experiments are in bold face (Heinz et al 1999).

One identified motif consisted of 10 nucleotides (`GNGCATTTGCNC`) and was detected immediately upstream of the minus 10 region. Two much shorter motifs were found close to the minus 35 region and several other identical nucleotides in the region flanking the -10 and -35 regions of both promoters (Figure 1). We speculate that the motifs identified in the two promoter regions are part of a possible *bio* box in *S. meliloti.* However, as currently only these two biotin regulated DNA loci in *S. meliloti* are identified it would be to preliminary speculate about a *bio* box consensus motif.

To our knowledge the *bioS* and the *bdhA* gene are the only DNA loci known to be transcriptionally regulated by biotin in *S. meliloti* and not linked to biotin synthesis. However, we speculate that *S. meliloti* might contain more then just these two DNA loci regulated by biotin. This hypothesis is supported by data from mutant screens using a transposable promoter probe. In one such screen we identified several novel DNA loci regulated by biotin (Streit, Phillips unpublished). However, *S. meliloti,* is probably not unique in its responsiveness to biotin, because *Rhizobium etli* produces 19 new proteins in the presence of biotin and thiamine (Encarnación et al 1989). Thus future work will focus on the isolation and characterization of novel biotin-regulated DNA loci in rhizobia.

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IMPORTANCE OF MOLYBDENUM AND COBALT TO THE BIOLOGICAL NITROGEN FIXATION

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1. Introduction

Results of strain selection studies regarding biological nitrogen fixation (BNF) were disguised by molybdenum (Mo) and cobalt (Co) deficiency. No differences were detected in soybean yield between plots without inoculation (1,870 kg/ha) and plots inoculated either with the combination of the strains SEMIA 587 + SEMIA 5019 (1,686 kg/ha) or SEMIA 5079 + SEMIA 5080 (1,715 kg/ha). However yield increased to 3.614 kg/ha when 200 kg of N-fertilisers were applied. At the same time, in one side experiment where Co + Mo were applied to the seeds with the inoculant yield increased by 44.2%. These results confirmed earlier experiments performed by Sfredo et al. (1997) and Campo & Lantmann (1998), indicating the importance of Mo on BNF in many tropical acid soils. Previously, Tong & Sadowski (1994) reported that Mo and Co application on the seeds, together with the inoculant, reduced bacterial survival on the seeds, nodulation and BNF efficiency. In sites of Paraná State, Brazil, Albino & Campo (1999), in laboratory and glasshouse experiments and Campo et al. (1999), in field experiments, confirmed that Mo and Co application on the seeds, just before inoculation, reduced bacteria survival, nodulation and BNF efficiency.

2. Aim

The aim of these studies was to confirm the importance of Mo and Co to BNF and to study an alternative method of application of Mo without reducing bacteria survival, nodulation and BNF efficiency.

3. Methodology

The experiments were set up in an oxisol (Latossolo Roxo distrófico) with a *Bradyrhizobium* population of 4.3×10^4 cells /g soil, with soybean cultivar BR 16, in a completely randomised block design with six replications. The micronutrientes $Na_2MoO_4.2H_2O$ (20 g/ha) and $CoCl_2.6H_2O$ (5 g/ha) were dissolved in 300 ml of a water plus 10% sucrose solution and applied to soybean seeds, just before inoculation, with a peat inoculant composed by the *Bradyrhizobium elkanii* strains SEMIA 587 + SEMIA 5019 (1.7×10^{10} cells/g inoculant). The different concentrations of Mo in the seeds were obtained by applying 400 or 800 g of Mo on the leaves 20 days after early flowering.

4. Results

Previous results showed that in soil without an established population of *Bradyrhizobium,* Mo + Co reduced nodulation and BNF. Glasshouse experiment showed decreases on nodule dry weight and N in grains of 21.8% and 7.8%, respectively. Results of two years experiments showed that in soils with established *Bradyrhizobium* population there were no effects of Co and Mo on soybean nodulation. But, in 1998, Co itself did not increase yield, Mo itself increased yield in 6% and Co +

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Mo increased yield in 20%. In 1999, Mo application increased dry weight of 100 seeds, N yield (18.5%) and grain yield (11.2%), but major effect were observed by the application of Mo + Co, increasing N yield (26.6%) and grain yield (14.5%).

There were no differences in yield between Mo application in soil or together with pre-sowing herbicides and the control, treatment without Mo. The application of Mo together with baculovirus or with insecticide, as well as post-sowing herbicides gave similar yield to the treatment without application of Mo to the seeds. The use of seeds with higher concentration of Mo (7.6 µg de Mo/g seed) plus Mo on the seeds yielded 5% more than normal seeds (0.14 µg de Mo/g seed) plus application of Mo on the seeds.

The use of seeds with high concentration of Mo did not increase nodulation but increased soybean yield. Seed poor on Mo (0.73 µg de Mo/g seed), without addition of Mo, yielded 2,314 kg/ha. Seeds with intermediate Mo contents (7.5 µg de Mo/g seed) yielded 3,167 kg/ha and seeds with high Mo concentration (13.3 µg de Mo/g seed) yielded 3,602 kg/ha of soybean. The use of seeds with high Mo concentration do not dismissed additional Mo on the seeds, but the needs of Mo were only 10 g of Mo when seeds with high concentration of Mo were used. The effects of Mo and Co on nodulation and BNF efficiency were also verified when seeds with different concentrations of Mo were used. There were no effects of Co application using seeds with higher or low Mo concentrations (0.73 µg de Mo/g seed or 13.3 µg de Mo/g seed). There were effects on weight of 100 seeds, N and grain yield when Mo was used alone, but the positive effects on BNF were higher when Co + Mo were used together

5. Conclusions

Results indicated that:

5.1 Application of Mo + Co on seeds reduces nodule dry weight and BNF in glasshouse experiment;

5.2 There are no Mo + Co effects on nodulation in soil with established *Bradyrhizobium* population;

5.3 Application of Mo and especially Mo + Co, increase BNF;

5.4 Application of Mo + Co on leaves have the same effect on BNF as seed applications;

5.5 Soil application of Mo + Co does not supply the Mo and Co necessary to the plant and to the BNF; and

5.6 Seeds with high concentration of Mo show higher BNF than those with low Mo contents.

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THE DIAZOTROPHIC CULTURE COLLECTION OF EMBRAPA AGROBIOLOGIA

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Embrapa Agrobiologia maintains an extensive Culture Collection of nitrogen-fixing microorganisms. These bacteria were isolated from many leguminous and non-leguminous plant species of economical and agricultural importance, from different regions of Brazil. This Culture Collection, registered at WDCC - World Data Center on Microorganisms under the number 364, has already a total of 1876 strains, characterized and catalogued, distributed on the following species: 71% of *Rhizobium, Bradyrhizobium, Mesorhizobium, Azorhizobium* and *Sinorhizobium,* 25% *Azospirillum, Herbaspirilium, Burkholderia, Acetobacter, Azotobacter* and *Beijerinckia* besides 4% of genetically engineered microorganisms (GEM's). Embrapa Agrobiologia Culture Collection strains are known by the BR identification. The main purpose of this Collection is to preserve rizobial strains recommended for commercial inoculant production and to provide these organisms, upon request, for many research studies, conducted at several national and international Institutions or Universities. The objective of this presentation is to analyse the request and supply service of the Embrapa Agrobiologia Culture Collection during the last 10 years.

The cultures are morphologically and biochemically characterized in the same culture medium used for isolation, and are preserved freeze-dried and also in tubes containing the culture medium used for growth, covered with a layer of sterilized liquid vaseline (Vincent, 1970). Rhizobia: Field isolates in yeast mannitol agar plates containing bromothymol blue (YMA) are tested for infectivity on the host-plant species in Leonard jars (Vincent, 1970). *Azospirillum, Herbaspirillum, Burkholderia, Acetobacter* and other associative diazotrophic bacteria, the cultures of these genera are isolated, characterized and authenticated as described by Döbereiner et al., 1995. Morphological characterization: change in pH of the medium during growth; time for colony formation; mucus production (quantity; consistency) and characteristics of the colony (size; form; color; transparency; elevation; surface; edge) as described by Yano et al., 1993.

The Embrapa Agrobiologia Culture Collection supplied a total of 2105 bacterial cultures during the period of January 1990 to June 1999. From these, 54% were sent to Brazilian Universities and Research Institutions and 46% to Institutions abroad. Brazilian requests (1,135) were concentrated on the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium* and *Azorhizobium.* Otherwise, only 31% of the international requests (970) were directed to this group of diazotrophic bacteria. International Research Institutions and Universities, especially from the American Continent (Argentina, Mexico, United States), are more interested in bacterial cultures of the genera *Azospirillum, Herbaspirilium, Burkholderia, Acetobacter* and other associative bacteria. The distribution of cultures supplied to the five Continents, shows a greater demand from the Americas which concentrated 78% of the requests with a total of 1,642 cultures sent in this period, followed by Europe (9%), Asia (7%), Africa (4%) and Oceania (2%).

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GREENHOUSE EVALUATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

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Crop rhizosphere has a high microbial activity because roots release substances which favour bacterium proliferation. The PGPR bacteria represent a large subgroup of microorganisms that colonize rhizospheres and promote plant development through metabolite production.

Most PGPR strains consist of Gram negative genera, and the greatest number of strains are members of the fluorescent *Pseudomonas* (Kloepper, 1993). Plant growth promotion by PGPR can result from one or more mechanisms including: biological control through competition, production of antibiotics or siderophores or for induced disease resistance and/or direct growth promotion through phytohormone production and increased nutrient availability through nitrogen fixation or organic and inorganic phosphate solubilization.

The aim of the present work is to confirm plant growth stimulation by these PGPR strains, used as bioinoculants in soybean and corn crops under greenhouse conditions.

Greenhouse experiments:

Crops: *Zea mays* cv. Dekalb 669-C5 and *Glycine max* cv. Buck Fulgor 33. For all experiments disinfected seeds were used.

Bacterial strains: In corn: 14 pure PGPR isolates and 9 combinations. In soybean: 12 pure PGPR isolates and 6 combinations, 1 *B.japonicum* C145 strain.

Flower pots with sterile soil and vermiculite at a 1:1 rate were used. Five sterile seeds were planted in each pot. Seedlings grew under the following conditions: soil humidity: 50% soil water holding capacity; temperature: 25-30° C during daytime and 14-16 ° C during nighttime.

Four days after sowing, the seedlings were inoculated with bacterial suspensions containing 10^8 cfu/ml, a non-inoculated control being used for corn and a control with *B.japonicum* for soybean. All soybean treatments, excepting the control, were inoculated with *B.japonicum* C145 and PGPRs.

The seedlings were watered with sterile water daily, and with Jensen's nutritive solution once a week. Observations were made all along the experience, measuring soybean and corn, stem and root length and weight, root volume and node weight in soybean. The experience was ended 50 days after planting .

Soybeans: According to the results, 12 treatments co-inoculated with C145+native PGPR yielded more dry matter than the C145 control.A point to be underscored is that the R44+N5 treatment surpassed the C145 control by 108% in dry matter production.The treatment bioinoculated with a pool of strains of 3 PGPR (Bt33+ BT35 +Btl5) surpassed the C145 control by 76%. Treatments N5, Bt5+Bt9 and Bt33+Bt35 showed a 53% increase of dry matter.Treatments 6,9, 10 and 12 had the largest node size and weight. Treatments 1, 3, 6, 9, 11 and 12 performed best with respect to root volume.

Corn: The assay was ended at day 50, stem and root length and dry weight of aerial biomass and root being assessed again 12 treatments surpassed the control in dry matter production, the most noticeable being isolates 20 and 21 with 27%; 9, 14 and 22 with 36%; and 11 and 17 with 45%.Twenty-three treatments surpassed the control in dry weight and 18 in root volume isolates 7 and 21 performing best.Results show that soybean and corn seed inoculated with native PGPRs permitted the seedlings a good establishment in the early crop stages and that in 60% of the treatments the interaction PGPR and *B. japonicum* bacteria / plant was positive.

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CHARACTERIZATION OF SALINITY-TOLERANTS *BRADYRHIZOBIUM SP* STRAINS

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Environmental stress plays an important role in crop yields of legumes grown in Latin America. Salinity is considered a governing stress factor in the normal distribution of plants and an increasing problem as the surface of unfavorable farming areas expands. Salt stress limits legume growth, especially when the crop relies on symbiotically - fixed nitrogen. Isolation and characterization of rhizobia strains tolerant to stress conditions, salinity among others, may allow to predict their eventual behaviour as a community in soils, and in this way may lead or not into a better interaction with the plant for its further introduction in unfavorable soils.

With the purpose of introducing *Cyamopsis tetragonoloba* (guar) in the semiarid areas of Argentina with problems of salinity we obtained salt- tolerants strains from the native symbiont *Bradyrhizobium* sp. GB2.
Salinity-tolerant *Bradyrhizobium sp* strains (88 mM, 264 mM and 440 mM NaCl) were characterized according to: Use of 95 sources carbonated by the BIOLOG GN method, type of metabolism, determination of exopolysaccharides, indol acetic acid production and/or analogues, siderophores production, phosphate solubilization, antibiotic resistance, plasmid profile and proteic and lipid profiles.

In our studies, 88mM - 264 mM NaCl tolerants significantly increase the ability to utilize different carbon sources with respect to the native strain, meanwhile tolerants to higher salinity show a sudden loss of utilization of tested carbon sources. Those strains that respond rapidly to the different carbon sources, root exudates among others, would have an advantage in the legume root rhizosphere over strains that respond slowly. *Bradyrhizobium* sp. GB2 showed slow growth rate, arriving to stationary phase 144 h upon incubation. Salt-tolerants strains showed rapid growth , arriving to stationary phase between 15 and 36 h upon incubation. Some tolerant strains lost a high-molecular-weight plasmid, suggesting that salinity may behave as curing agent. Qualitative and quantitative changes were observed when total lipids, proteins and carbohydrates were determined. This makes them to exhibit physiological and metabolic behaviours different from the native strain , for instance the capacity of taking up different compounds or the resistance to antibiotics, wwith the exception ampicillin, an apparent chromosome marker.

In general, the salinity-tolerant strains increased their adaptive capacity when compared to the native strains by showing larger C-sources-oxidation capacity, increase in growth rate and production of exopolysaccharides involved in adherence. Tolerant strains are efficacious in nodulating up to 254 mm-NaCl-salinity range and some of them have acquired PGPR capacity being therefore promising strains for unfavorable areas.

LEGUMINOUS-*(BRADY)RHIZOBIUM* INTERACTION IN PRESENCE OF THIRAM FUNGICIDE

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Under soil N-limiting conditions, bacteria of the familia *Rhizobiaceae* cause the formation of specialized organs (nodules) in roots and stems of legume plants. In these structures, bacteria convert atmospheric N_2 into ammonium wich is employed by the plant as a N source.
Reports suggesting that fungicides such as thiram used as seed protectors might affect the leguminous-rhizobia symbiosis have been known since the 1960's. The purpose of this work is to study the effects of thiram on the processes of plant-bacteria recognition and the initial nodulation states in soybean and alfalfa as well as the selection of fungicide-resistant bacterial strains to be used in inoculant formulation.

In the early stages of the symbiosis, flavonoid compounds present in exudates of legume roots and seeds cause the expression of bacterial genes (called *nod* and *nol*) thus initiating the nodulation process. The capacity of seed and root exudates and pure flavonoids to induce nodulation genes was evaluated using isogenic strains of rhizobia (obtained from S. Zaat, Leiden University, Netherlands) where promoters of the *nod* area were cloned with the structural gene *lacZ* from *Escherichia coli.* Exudates obtained from seeds treated with thiram (and their controls without fungicide) were utilized without purification and pH was adjusted to 7. Then, 500 μl of exudates were combined with 500 μl of strain culture and incubated at 28°C for 3 h. In the assay with pure flavonoids, these were used at a concentration of 10 μM and thiram was added at a final concentration of 2 mg/ml. Released β-galactosidase activity was estimated by Miller's method (1972).

The results show that thiram significantly inhibits the induction of bacterial nodulation genes. The quantity or quality of vegetal exudates could be affected as well as their interaction with the *nodD*-gen product (Andrés et al. 1998 a)

Bradyrhizobium japonicum strains highly resistant to thiram were also obtained in this work. Some of these strains (*TIIB, A86* and *A2*) keep their symbiotic properties intact. In other strains, resistance acquisition led to the loss of nodulating capacity (strains *A1, C1* and *C6).* Characterization tests such as growth at different pH, denitrifying ability, salt tolerance, production of siderophores and phosphate solubilization were performed on the resistant strains. Inoculants produced from these strains could be appropiate for use with thiram treated seeds (Andrés et al. 1998 b)

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CHARACTERIZATION OF ANTIFUNGAL AGENTS PRODUCED BY *PSEUDOMONAS AURANTIACA* FOR ITS USE AS BIOCONTROL INOCULANT.

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Losses in vegetalbe production caused by fungal diseases are an important economic factor to be considered. Today, one of the most outstanding techniques for phytopathogenic fungi control consists in using organisms capable of producing substances such as antibiotics, siderophores and cyanids which limit the development of pathogens (Glick , 1995). *Pseudomonas* is one of the most used genus due to its abundance in the rhizosphere as well as its prolific production of secondary metabolites involved in phytopathogenic control (Défago and Haas, 1990; O'Sullivan and O'Gara, 1992). *Pseudomonas aurantiaca* is a bacterium found in soybean rhizosphere in the region of Río Cuarto, Córdoba, Argentina. *In vitro* tests, using different culture media, reveal an excellent biocontrol capacity over *Macrophomina phaseolina, Rhizoctonia solani, Fusarium sp., Phytium sp.* and *Alternaria sp.* This biological activity is closely related to the secretion of an orange coloured pigment into the culture medium, wich appears at about 48 hs from the start of growth in agar trypticase soybean (TSA). At this time, the biotests with *M. phaseolina* show a 68 % inhibition of fungal growth . The antifungal effect and the intensity of the pigment colour are weaker in nutritive agar (NA), while in minimum medium (MM) pigment production is not apparent but the antifungal activity on the aforementioned species is 41.8 %. These results suggest the participation of more than one bacterial metabolite in biocontrol.

The addition of tryptone to MM induces the production of antifungal pigment, not being this the case when suplementing with arginine, methionine, histidine and tryptophan. When adding glucose in concentrations above 5g/l to TSA, the pigment intensity and the antifungal effect decrease. The suplementation with $FeCl_3$ does not have an effect on pigment production, but a lower percentage of fungal inhibition is asserted; this indicates that one of the antifungal metabolites would have a siderophore function. The antifungal activity of the sterile supernatant of a bacterial culture in TSA medium is resistant to treatment with proteases (trypsin and K proteinase), to pHs from 4.5 to 12 and to the effect of temperature (100°C, 20 min). This stability suggests that the bioactive principles do not have a proteic nature. The extraction of the bacterial metabolites with antifungal activity was done in organic solvents (acetone and ethyl-ether). Using the dry residue (raw extract), a thin layer chromatography was performed; this separation reveals two fractions with biologic activity, when chloroform is used as running solvent, the Rf values are 0,35 and 0.37 respectively. The first one inhibits the development of the mycelium of *M. phaseolina* by 48 %, while the second one obtains a 40% inhibition. A synergical effect is noticed when the biotest is done with the mixture of both fractions (60% inhibition). *P. aurantiaca* can coexist with *Rhizobium meliloti* and *Bradyrhizobium japonicum* strains; the bioactive compounds do not interfere with the normal growth of these microorganisms, which play an important symbiotic role in cultivars such as alfalfa and soybean.

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CHICKPEA RESPONSE TO INOCULATION AND NITROGEN FERTILISATION: WINTER AND SPRING VARIETIES

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Chickpea is considered as an alternative crop to cereals under the climatic conditions of the Mediterranean region of Europe, specially after the development of resistant varieties to cool conditions and to the pathogenic fungi *Ascochyta rabiei,* making possible its use as a winter crop with advantages in yield potential (Sousa, et al.,1990). Results for grain yield from field trials at different locations in Portugal indicate an average of 2500 kg ha^{-1} for varieties sown in autumn and 700 kg ha^{-1} for varieties traditionaly sown in spring (Duarte, et al.,1992). However, this high yield potential of the winter forms of chickpea seems to be highly site dependent.

Chickpea response to inoculation is tested under laboratory conditions. Winter and spring forms were used to test the symbiotic efficiency of local and introduced strains. The efficiency of strains under lab conditions was depending on the variety and was generally low. The introduced strains were less efficient than the local ones, both for winter and spring forms, which is in accordance to Sattar et al.(1995).

Under field conditions only a winter form of chickpea was tested . In two consecutive years of trials, there was no crop response (grain yield and total biomass) to inoculation for the two locations tested and the crop response to mineral N fertilisation (0, 25 and 150 Kg Nha^{-1}) was very limited and it happened only at one site. The lack of response to inoculation is in accordance with the lab results. However the small response to N fertilisation was unexpected. This can be due to some limitations of chickpea plants in mineral nitrogen assimilation under field conditions, as suggested by Herridge et al.(1995) and Bonfil et al.(1995), making the nitrogen uptake by this species highly dependent on nitrogen fixation. Therefore the lack of response observed in this experiment could be due to the absence of any highly specific strain, both in the native population and in the inoculant strains used. The selection of more efficient strains is still needed to fully take advantage of the high yield potential of the winter forms of chickpea.

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OCCURRENCE AND DISSEMINATION OF ENDOPHYTIC DIAZOTROPHIC BACTERIA IN SUGARCANE FIELDS.

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Acetobacter diazotrophicus, Herbaspirillum spp. and *Burkholderia* spp. are the bacteria found most frequently associated with sugarcane plant and studies have shown that they survive very poorly in soil (Baldani et al., 1997). However, such bacteria could be isolated from micro-propagated plants growing in these soils, suggesting a viable but non cultivable form when the host plant is absent (Reis et al., 1998). We have searched for the occurrence of these species in different soils (Rio de Janeiro and São Paulo) planted to sugarcane as well as in sugarcane trash and roots. So far, we have only found *Burkholderia* spp. in soil samples when micro-propagated plantlets were used as a trap host. On the other hand, *A. diazotrophicus,* but not *H. seropedicae,* could be isolated from trash harvested from sugarcane fields. The presence of *A. diazotrophicus* and *H. seropedicae* in soil could not be detected using the species-specific primers, suggesting that these bacteria were absent in the soil, or the PCR method was not sensitive enough to detected the bacteria. Kirchhof et al (1998) found a detection limit of 200 cells/reaction when *A. diazotrophicus* was present in the plant tissue samples. In addition, soils were inoculated with cane residues (leaves or roots) and planted with micro-propagated sugarcane to determine the role of cane residues in the dissemination of these bacteria in the field. The preliminary results showed that the three species (*A. diazotrophicus, Herbaspirillum* spp and *Burkholderia*) could be detected in the plants grown in soil containing these residues. The results suggest that plant residues play a role in the survival and dissemination of these endophytic diazotrophic bacteria in the soil.

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GENOMIC FINGERPRINTING OF *BRADYRHIZOBIUM JAPONICUM* ISOLATES BY RAPD AND REP-PCR

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Genetic diversity of indigenous *Bradyrhizobium japonicum* population in Croatia was studied by using different PCR-based fingerprint methods. Characteristic DNA profiles for 20 *B. japonicum* field isolates and two inoculant strains were obtained using random primers (RAPD) and two sets of repetitive primers (REP- and ERIC-PCR). In comparison with the REP, the ERIC primer set generates fingerprints with a lower level of complexity, but still several specific bands were detected. Different *B. japonicum* isolates could be more efficiently distinguished by using combined results from REP- and ERIC-PCR. The most polymorphic bands were determined in amplification reactions with four different RAPD primers. Both methods, RAPD and rep-PCR, resulted in identical grouping of strains. Cluster analysis, irrespective of fingerprint method used, revealed that all the isolates could be divided into three major groups. Only different degree of relative similarity between these groups and a minor rearrangement of strains within major groups were determined depending upon the method used. Our results indicate that both RAPD and rep-PCR fingerprinting could effectively distinguish different *B. japonicum* strains. RAPD fingerprinting proved to be slightly more discriminatory than rep-PCR.

NODULATION CAPABILITY OF SPECIES OF *STYLOSANTHES* AND *AESCHYNOMENE* BY RHIZOBIA FROM THE BRAZILIAN SEMI-ARID REGION

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The *Aeschynomene* tribe, which includes the genera *Arachis, Stylosanthes* and *Aeschynomenae,* are described as being nodulated by a large range of rhizobia (miscellany cowpea or tropical rhizobia). Tropical rhizobia display a large phenotypical and genotypical diversity and they are capable of nodulating several species of leguminous plants (Dadarwal et al, 1974; Ligon et al., 1982). It is not a homogeneous group and differences related to BNF efficiency have been described (Wong et al. 1988). Thies et al (1991) suggested that peanut rhizobia should be a subgroup within the tropical rhizobia. The differences among the plants regarding host ranges have also been reported related to cultivars or species. Chandapillai (1972) studying 7 *Stylosanthes* species inoculated with 25 rhizobial strains found a variation in nodulation efficiency depending on the association. For a long time, it has been emphasized the need of an adequate strategy for selection of a specific strain to be used as inoculant (Mannetje, 1969, in: Allen, Allen, 1981). The aim of this work is to investigate the nodulation capacity by native rhizobia strains of *Stylosanthes* and Aeschynomenae species cultivated in soil samples from the North-east Region. The nodulation capability of the following leguminous species were investigated: *Stylosanthes guianensis* (cv.Bandeirantes) *S. scabra* (cv. Seca), *Aeschynomenae americana* (cv. Nova Odessa), *A. paniculata* (cv. IZ-No-SP) and *A. histrix* (cv CIAT-8225). Sterilized seeds were grown in pots with soil samples at the following sites: Site 1 - Caruaru (peanut crop area); 2 - Serra Talhada (Stylosanthes crop area); 3 - Serra Talhada (peanut crop in traditional agricuture); 4 - Serra Talhada (*Leucaena leucocephala* and *Mimosa caesalpinifolia* crop area); 5 - Serra Talhada - Caatinga area (native vegetation). The results showed that *S. guianensis* was capable of nodulating with rhizobia present in 4 of the soil samples used while *S. scabra* (cv. Seca) was nodulated by strains from only 2 soil samples (Site 2 and 5). The three species of *Aeschynomene* showed a restriction nodulation pattern. Each of them nodulated with native rhizobia found only in 1 soil sample. In Site 2, where *Stylosanthes* is cultivated, the native rhizobia population was capable of nodulating the majority of the species used, suggesting that a rhizobial population specific for these tribe was selected. The results presented showed that *Sthylosanthes* and *Aeschynomenae* do not possess a broad host range, suggesting that another sub-group within tropical rhizobia may be defined as Thies et al. (1991) have already proposed for peanut. Furthermore, a strategy for optimizing inoculant selection, through selecting specific associations may render better response to inoculation.

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NATURE OF THE BENEFICIAL *RHIZOBIUM LEGUMINOSARUM* BV. TRIFOLII – RICE ASSOCIATION

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Two cycles of field and laboratory studies have indicated that a diverse population of the clover root-nodule symbiont, *R. leguminosarum* bv. trifolii intimately colonizes rice roots in the Egyptian Nile delta where this cereal has been rotated successfully with berseem clover (*Trifolium alexandrium*) since antiquity (Yanni et al. 1977; Yanni et al. unpublished). A few isolates from this natural bacterial-plant association display significant potential to promote rice growth under laboratory, greenhouse and field conditions. This plant growth-promotion response exhibits strain/variety specificity. Some of these rice-adapted rhizobia induce an expansive root architecture and increased uptake of several plant nutrients in culture with rice, and can extracellularly solubilize precipitated phosphate and secrete indoleacetic acid and gibberellic acid phytohormones *in vitro.* ARA and ^{15}N-based studies (^{15}N natural abundance, ^{15}N isotope-dilution) do not support a major role of BNF in this plant growth-promotion response. Two field inoculation trials conducted in Egypt have revealed statistically significant increases in rice grain yield and agronomic fertilizer N-use efficiency due to inoculation with certain rhizobial strains; a third field inoculation trial is in progress. Microscopical studies reveal that these rhizobia can colonize the rice root surface and enter the root interior through cracks at lateral root emergence and at junctions between epidermal cells, where they exhibit some internal root colonization (Prayitno et al. 1999). We have created an international network of research collaborations focused on continuing basic and applied studies of this newly described *Rhizobium*-rice association. These studies should help to identify beneficial rhizobia that have already adapted to rice and can be manipulated to increase its productivity with less dependence on fertilizer-N inputs. This *Rhizobium*-rice association represents a unique experimental system that has significant potential to benefit sustainable rice agriculture where the goal is to enhance the crop's output without irreparably damaging the natural resource base where rice can be grown.

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INFECTIVITY AND COMPETITIVENESS FOR NODULATION OF N-LIMITED *BRADYRHIZOBIUM JAPONICUM.*

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1. Introduction.

Soil combined nitrogen scarcity is a prerequisite for legumes nodulation by rhizobia. While inhibitory effects on nodulation are exerted in the plant by the abundance of this nutrient, nitrogen limitation has been observed as stimulatory of bacterial symbiont infectivity (Brelles-Mariño, Boiardi, 1996). Here we report on the effects that *B. japonicum* growth on nitrogen-limited media has on its infectivity and competitiveness for soybean nodulation.

2. Materials and Methods.

We obtained the strains LP 3001 (Spc-resistant) and LP 3004 (Str-resistant) as spontaneous derivatives from USDA 110. Antibiotic resistance was stable after passage through nodules. Both strains were identical to parent strain, and between them, in their nodulating and competitive abilities. These rhizobia were grown in liquid Götz et al. (1982) media either complete (N100), with N-source reduced to 10% (N10) or without N-source (N0). Infectivity was measured as the distribution of nodules along the primary soybean roots with reference to the position of the root tip at the time of inoculation, in plastic growth pouches (Bhuvaneswari et al., 1980). Competitiveness was assessed by recording nodule occupancy of soybeans cultivated in vermiculite pots inoculated with 3001/3004 mixed suspensions.

3. Results and Discussion.

Logarithmic phase of growth lasted for 10 days, entering stationary phase around 11-12 days. Survival at 23 days of culturing was higher in N0 and N10 than in N100. For each one of the three N-conditions in independent experiments, logarithmic growth phase cultures inoculated in plastic growth pouches gave around 50% more nodules and their distribution was more concentrated around the root tip position at the time of inoculation, than stationary-phase cultures thus showing a higher infectivity. When the effect of N-limitation was compared in LP 3001 cultures of the same age, we observed that for logarithmic (5 days) cultures, N0 and N10 were significantly more infective than N100. For stationary (14 days) cultures there were no significant differences. Again, logarithmic cultures were always more infective than stationary ones.

In competition, logarithmic cultures occupied significantly more nodules than stationary cultures only when grown in N10. To assess competition against an established population, we inoculated a rhizobial suspension of $1.2\ 10^5$ CFU ml^{-1} LP 3004 cells in vermiculite pots. After 30 days in the green house, the population grew to $1.4\ 10^7$ CFU/ ml substrate solution, thus showing a remarkably higher survival than in the liquid media. At this time we planted soybeans in these pots, inoculating each plant with 10^8 to 10^9 CFU of either logarithmic (5 days) or stationary (14 days) LP 3001 cultures, each grown in N0 or N10 or N100. After 25 days, more than 66% of the nodules were occupied by LP 3004 in all the cases, and competitiveness of all the LP 3001 cultures was statistically similar, independently of the culture age or N-level in the media. Therefore, enhanced infectivity of young, N-limited *B. japonicum* was not translated to an increase of competitiveness.

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GENOTYPIC DIVERSITY OF RHIZOBIA NODULATING COMMON BEANS *(PHASEOLUS VULGARIS* L.) IN BRAZILIAN TROPICAL SOILS

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The common bean, *Phaseolus vulgaris* L., is considered a poor N_2-fixing plant because many factors influence performance in the field. One limitation is the plant's promiscuity, which nodulates with a wide range of rhizobial species, and its sensitivity to stresses, especially high soil temperatures and acidity. The rhizobia that infect common beans in tropical soils belong to three main species: *R. leguminosarum* bv. phaseoli (Jordan, 1984), *R. tropici* (Martinez-Romero et al, 1991) and *R. etli* (Segovia et al, 1993). The ecological importance of *R. gallicum* and *R. giardinii* (Amarger et al, 1997) in tropical soil has not yet been established. Previously, we studied the genotypic diversity of bean-nodulating rhizobia present in tropical soils frequently submitted to high temperatures (Straliotto et al, 1997). Strains were isolated from root nodules harvested from *Phaseolus vulgaris* and *Leucaena leucocephala,* inoculated with soil samples from different bean cropping areas, and grown in Leonard jars incubated at low and high temperature regimes, as previously described. The objective was to select better adapted strains by evaluating the symbiotic efficiency and heat tolerance of the most representative groups obtained by Straliotto et al. (1997) through a genotypic analysis of diversity based on ARDRA. The symbiotic efficiency of the representative strains from each cluster were compared to strain BR855 (*R. tropici* IIB) since it has a symbiotic performance similar to the efficient strain CIAT 899 at normal growth conditions, but being symbiotically more heat tolerant. *Leucaena* and bean isolates were tested for nodulation of bean plants (cv. Carioca). Duplicate Leonard jars were inoculated with 1 ml of a 3-day-old pure cultures of rhizobia grown in YM medium. Bean plants were harvested 30 DAE, and evaluated for nodulation and efficiency (shoot dry matter accumulation). The most efficient strains from each of the main bean-nodulating groups were evaluated for heat tolerance in symbiosis with beans. Plants were grown in Leonard jars, immersed in a water bath which temperature was controlled by thermostats to allow a heat shock treatment (38°C, 5h during 3 days) at 30 DAE. Plants were harvested 59 DAE and evaluated for shoot dry matter and nitrogen accumulation. Most of the isolates recovered from the traditional bean cropping areas analysed belonged to the groups of *R. tropici* IIA, *R. leguminosarum* bv. phaseoli/*R. etli* and *Sinorhizobium* spp. The isolates recovered from *Leucaena* as trap host were mainly classified as *R. tropici* IIA and *Sinorhizobium* and showed greater symbiotic efficiency when inoculated on beans. On the other hand, most of the isolates recovered from beans incubated at the lower temperature regime, belonged to the *R. leguminosarum* bv. phaseoli/*R. etli* cluster, with a greater percentage of strains of low efficiency or even inefficient on beans. The heat shock experiments showed that it is possible to select heat tolerant strains from all the bean-nodulating groups of rhizobia. Some strains were more efficient in translocating nitrogen to the pods after heat shock, which is an important characteristic for the selection process.

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PCR/RFLP ANALYSIS OF *ACETOBACTER DIAZOTROPHICUS* STRAINS ISOLATED FROM SUGARCANE GENOTYPES ORIGINATING FROM DIFFERENT COUNTRIES

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Acetobacter diazotrophicus is a Gram-negative, rod-shaped, N_2-fixing microaerophilic bacterium that is found mainly associated with sugar-rich plants, such as sugarcane, sweet potato and Cameroon grass, all of which can be propagated vegetatively (Cavalcante and Dobereiner 1988). The low genetic diversity of this bacterium could be explained by its recent evolution or limited habitat (Caballero-Mellado et al., 1995), however, Brazilian isolates are more heterogeneous genetically than the Mexican ones and this may reflect differences in nitrogen fertilizer doses applied to sugarcane crops in these countries (Caballero-Mellado et al., 1995). However, no study has considered the origin of the plant on the genetic diversity of *A. diazotrophicus.* Forty six isolates were obtained from leaves and roots of sugar canes varieties originating from different countries (15 from Brazil; 9 from India; 13 from USA; 3 from Barbados; 2 from Cuba; 1 from Puerto Rico;l from Argentina and 2 from Mexico) and maintained at a Germoplasm bank, for about 30 years. Seven type strains were also analysed. The genetic diversity was determined by amplification of the 16S – 23S rDNA IGS using primers pHr and p23Suni322anti (Massol-Deya et al., 1995; Honeycutt et al., 1995). PCR/RFLP analysis were performed for six restriction endonucleases. The results (Fig. 1) showed that *A. diazotrophicus* strains analyzed were grouped into two subcluster without the influence of plant origin or part of the plant (root and leaf) from where these bacteria were isolated. These results reinforce the idea of limited genetic diversity of this species as suggested by the multilocus enzymes analysis (Caballero Mellado et al., 1995).

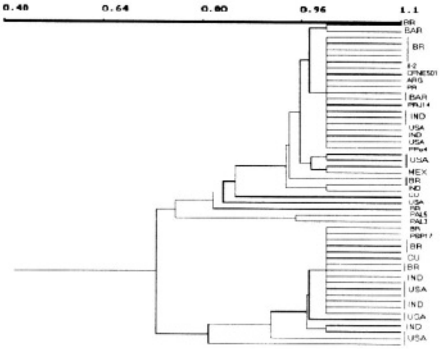

Figure 1 - Similarity dendrogram based on PCR-RFLP of the 16S – 23S rDNA–IGS of *A. diazotrophicus* strains isolated from sugarcane of different countries. (BR – Brazil; IND – India; USA - United State; BAR – Barbados; CU – Cuba; PR – Puerto Rico; ARG – Argentina; MEX – Mexico.
Reference strains: PAL5, PAL3, PPe4, PRJ14, PSP17, CFNE501 and 8-2.

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COMPATIBILITY BETWEEN *RHIZOBIUM* STRAINS AND RECOMMENDED FUNGICIDES FOR COMMON BEAN(*PHASEOLUS VULGARIS* L.)

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Seeds of common bean are often treated with fungicides to prevent the occurrence of soil-born diseases. There are few studies showing the compatibility between *Rhizobium* inoculation on seeds and use of fungicides.

The objectives of this work were: (1) to study the compatibility between recommended strains of *Rhizobium* for bean inoculation and recommended fungicides applied to the seeds; (2) to study natural resistance of *Rhizobium* strains isolated from cerrado soils, which nodulate common bean plants.

Strains CIAT 899 and PRF81-81 were grown on YEM media and transferred to sterilized peat. Strains were inoculated on bean seeds at the recommended dose of 600 g/50 kg seeds. Before inoculation, seeds were treated with fungicides and immediately after inoculation, the number of viable cell was counted by dilution method in Petri dishes. The following fungicides were used (g/50 kg seeds): benomyl (50g), captan (100g) and thiabendazole (50g). The number of cells for untreated seeds was: 6.6x10^{6} and 13x10^{6} for CIAT 899 and PRF-81, respectively.

Immediately after inoculation (time 0), benomyl promoted mortality of 71% to 86% for CIAT 899 and PRF-81, respectively (Table 1); for captan, the mortality ranged from 37 to 44% and for thiabendazole, 37 to 15%, for CIAT 899 and PRF-81, respectively. *In vitro* tests with 30 native rhizobia isolated from cerrado soils showed that only one isolate was able to grow up to 150 ppm of captan. For thiabendazole, 70% of the isolates were grown at 400 ppm and 40% at 800 ppm. For benomyl, 78% were able to grow at 400 ppm and 30% were grown at 1000 ppm.

Table 1. Compatibility between strains PRF-81and CIAT 899 with three fungicides, at recommended dose. Means of three replicates.

Treatment	CIAT 899		PRF-81	
	UFC.seed^{-1}	% Mortality	UFC.seed^{-1}	% Mortality
Control	13,3x10^{6}a	-	6.6x10^{6}a$^{(1)}$	-
Benomyl	3.99x10^{6}b	70.73	0.89x10^{6}b	86.52
Captan	8.57x10^{6}ab	37.13	3.70x10^{6}ab	43.94
Thiabendazole	8.48x10^{6}ab	37.79	5.57x10^{6}ab	15.61

(1) Numbers followed by the same letter in each column, do not differ statistically by Tukey's test($p<0.05$).

RESPONSE OF FIELD GROWN BEAN (*PHASEOLUS VULGARIS* L.) TO *RHIZOBIUM* INOCULATION AND N FERTILIZATION IN TWO CERRADOS SOILS

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Most soils sown to field beans (*Phaseolus vulgaris,* L) contain indigenous rhizobia which might interfere with the establishment of the inoculated strain. As a consequence, the benefits of bean inoculation are usually questioned and the use of N fertilizer is gradually becoming a common practice. The present study had the objective of evaluating the effectiveness of inoculation and N fertilization in field soil with (site 1) and without (site 2) a previous bean cropping history. Inoculation response was variable in the two areas. In site 1, with a bean rhizobia population of 7×10^2 cells g^{-1} soil, plants were well nodulated in the control treatment and inoculation had no effect in any of the parameters evaluated. The serological analysis confirmed that the inoculated treatment failed to increase the nodule occupancy by strain CIAT 899 in nodules and that the strain inoculated in the previous bean crop, UMR 1135, also had a poor establishment in the soil. Consequently, in this experiment 90% of the nodules were occupied by indigenous strains. Although CIAT 899 was used as a highly enriched inoculum (10^9 cells g^{-1}), the strain was not able to compete against the soil rhizobia. In contrast, in site 2, which had not been previously cropped with bean and showed a bean rhizobia population of less than 10 cells g^{-1} of soil, non-inoculated plants were poorly nodulated at 15 days after emergency. Inoculation had a statistically significant effect on nodulation parameters at 15 and 35 days and on grain yield as well as increased the occurrence of the inoculated strain in nodules from 0 to 91 %. In site 1 (previously cultivated with bean), application of N fertilizer (starting 14 DAE) had no effect on early nodule formation (number of nodules at 20 DAE), but decreased nodule number at 40 DAE and nodule dry weight at 20 and 40 DAE, particularly at high levels of N. Nitrogen fertilizer also increased both shoot dry matter of plants at 40 and 60 kg N ha^{-1} and grain yield, particularly with 60 kg of N ha^{-1}. In site 2, statistical differences on nodulation and grain yield were not detected due to the application of N fertilizer, although nodule dry weight at 40 DAE decreased in up to 55% under the high levels of mineral N. There was a statistically significant interaction between mineral N and nodule occupancy by strain CIAT 899, and by the indigenous strains (nodules without reaction) in site 2. In the inoculated plots, increasing levels of N fertilizer decreased nodule occupancy by the inoculated strain CIAT 899 from 91 to 17%. Contrarily, nodule occupancy by the indigenous strains (nodules without reaction) followed an opposite trend indicating that the latter are more tolerant to mineral N. Those indigenous strains may be a valuable source in a selection program of rhizobia strains tolerant to mineral N. The results indicate that the response to inoculation and N fertilization differs according to cropping history. When bean is cultivated for the first time, indigenous rhizobial populations are low and yields above 3,000 kg ha^{-1} may be accomplished exclusively with seed inoculation, with no further response to N fertilizer levels as high as 80 kg N ha^{-1}. In contrast, previous cultivation of bean increases indigenous soil rhizobia and prevents nodule formation by the inoculated strain, therefore N fertilizer may be necessary to reach maximum yields. In this case, no benefits were obtained by the combination of inoculation and N fertilization, but this practice may be feasible with the selection of N tolerant strains and appropriate strategies of N fertilizer management.

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GENETIC DIVERSITY OF FAST GROWING RHIZOBIA ISOLATED FROM DIFFERENT GEOGRAPHICAL AREAS.

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Fast growing rhizobia that induce nodules on soybean were isolated from China, Vietnam and Papua New Guinea (Balatti, 1994). These organisms were partially characterized by several authors based on physiological and symbiotic characteristics (Sadowsky et al., 1983; Cleyet-Marel, 1987, Navarro et al., 1996) Several evidences suggest that some of the fast growing isolates might be evolutionary intermediates Therefore, the aim of this project was to fingerprint the genome of fast growing organisms by different PCR methods to analyze the genetic relationship of these organisms.

DNA was extracted from fast growing strains isolated from the soils of China, Vietnam and Papua New Guinea, respectively. We fingerprinted the microorganisms genome with Enterobacter Repetitive Intergenic Consensus (ERIC), Repetitive Extragenic Palindromic elements (REP) and Random Amplified Polymorphic DNA (RAPD) primers. Also, amplified 16SARN and Intergenic Sequence (IGS) *nifH-nifD* DNA fragments were analyzed by restriction analysis with *Alu* I, *Hae* III, *Rsa* I, *Hind* III and *Hinf* I. UPGMA algorithm was used to perform a hierarchical cluster analysis. Pairwise comparisons were calculated using Simple Matching (SM) coefficient. Principal coordinate Analysis (PCA) was also performed.

The analysis of ERIC, REP and RAPD fingerprints revealed considerable diversity between fast growing rhizobia and confirmed the results of Perret et al (1994) that these are closely related organisms. However, these fingerprinting techniques suggest that *Rhizobium* sp. NGR234 is more closely related to *S. fredii* strains, than at least some *S. f* strains are between each other. In addition the analysis of the results suggest that the strains isolated from the soils of Vietnam were the most similar *S. fredii* with a SM coefficient of 90%.

ARDRA analysis of the 16SRNA and IGS *nifD-nifH* suggest that *R.* sp. NGR 234 is, as expected, though closely related to *S. fredii* isolates, the most distantly related organism. Cluster analysis performed either with results from ARDRA or from REP, ERIC or RAPD, grouped the strains based on the place of origin. Though more work has to be done the similarity coefficient found between *S. fredii* strains and *Rhizobium sp.* NGR234 suggest that this strain should most probably be named *Sinorhizobium fredii.*

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NITROGEN FIXATION, COMPETITIVENESS AND SURVIVAL ON LIQUID AND PEAT BASED INOCULANT OF FAST GROWING STRAINS *SINORHIZOBIUM FREDII*

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Fast growing rhizobia *Sinorhizobium fredii* nodulates an ample number of legumes (Pueppke and Broughton, 1999). Most strains nodulate and fix nitrogen on soybean as efficiently as *Bradyrhizobium japonicum* (Buendía-Claveria *et al.*, 1994; Videira *et al.,* 1999). Therefore, the purpose of this project was to compare the ability of *B. japonicum* and *S. fredii* strains to fix nitrogen, compete and survive on commercial inoculants and when they are introduced in new environments.

Bradyrhizobium japonicum and *S. fredii* grew on pH 6.00 to 8.00 but not lower. Only *S.f.* HH102 was sensible to pHs equal or lower than 6.5. A temporal exposure to pH stress did not affect *B.japonicum* and *S.fredii,* except for strain HH102 which proved to be susceptible to pH stress.

Metabolic differences were found between *S. fredii* strains since different media had to be used to obtain cultures of the strains with high cell numbers, so that they can be used in inoculant production. Cell survival on peat and liquid formulated inoculants was tested by counting the Colony Forming Units on Yeast Extract Mannitol plates, when they were inoculated with serial dilutions of the inoculant. Either on peat based or liquid inoculants, two *S. fredii* strains HH102 and HH103 were unstable while two other ones, SMH12 and USDA191, survived at high numbers even 180 days after production.

We identified a PCR fingerprinting method that allowed us to discriminate between *S. fredii* and *B. japonicum.* We used two 20 bp primers with homology to the *nolXWBTUV* locus. These primers generated a 240 bp fragment with *S. fredii* template DNA and a 350bp fragment with *B. japonicum* template DNA. We succesfully identified bacteria inside nodules colonized by *S. fredii, B. japonicum* and also nodules occupied by both bacteria. We are currently studying with this tools *S. fredii* population dynamics when they are introduced in new environments. Our results show that *S.fredii* strains are good nitrogen fixers and that, when suspended in liquid or peat based inoculants, they have survival capabilities similar to *B. japonicum.*

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STABILIZATION AND SURVIVAL OF *RHIZOBIUM MELILOTI* SUSPENSIONS IN RELATION TO THE EXOPOLYSACCHARIDE AND BIOMASS CONTENT

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Extracellular polysaccharide production by *Rhizobium meliloti* strains can be related to the culture medium (Van Workum, 1998). Here, we consider, the influence of aeration rates on biomass and exopolysaccharides formation, using a typical medium (Balatti, 1996). Our purpose was to study the effect of different exopolysaccharides concentrations on the stabilization and survival rates of *Rhizobium meliloti* suspensions. High survival rates and retaining their symbiotic properties are principal characteristics of a good liquid *R.meliloti* inoculant. There is limited information on the cell stabilization. The addition of the protein and xanthan gum are indicated but the use of *Rhizobium* polysaccharide is not mentioned. We considered how to obtain high cell concentration of *R.meliloti* with different levels of exopolysaccharides achieved at different aeration conditions. The *R.meliloti* strains isolated in our laboratory were preserved by subculture in medium contained manitol, agar, at 5°C. The volume of inocula was 5 - 10% of the volume used in the fermentation process. The initial concentration was about $5x10^8$ cell.ml^{-1}. The medium contained (g.l^{-1}): saccharose, 10; yeast extract, 4; KNO_3, 0,8 and salts. The bacterial growth and survival rate were determined by viable cell count (Kotch, 1994). The exopolysaccharide concentration in the culture was determined by precipitation with ethanol (Hebbar, 1992). The experiments were performed in erlenmeyer at 28°C in a rotary shaker at 250 rpm and 2,5 cm of stroke. The aeration rates of 200 to 500 ml O_2.l^{-1}.h^{-1} (Pirt, 1975) were established using different volume of culture in relation to volume of flask. The obtained cell and polysaccharide concentration were in the order of 4-5 g.l^{-1} and 3-11 g.l^{-1} respectively. To prepare the stabilization suspension we used different cellular biomass and exopolysaccharide concentrations adding of 0,5 M of saccharose for the osmotic balance and 0,3 g.l^{-1} KH_2PO_4 and 0,6 g.l^{-1} Na_2HPO_4 to regulate the pH. With reference to the used conditions the obtained of the exopolysaccharide concentration is influenced by the aeration levels of the culture medium. We observed for this strains, that the highest concentration of exopolysaccharide is obtained with low levels of aeration rates, for example at 529 ml O_2.l^{-1}.h^{-1}, the polysaccharide concentration was 4 g.l^{-1} and at 367 and 323 ml O_2.l^{-1}.h^{-1} the exopolysaccharide concentration were 6 and 7 g.l^{-1} respectively.

Suspension of *Rhizobium meliloti* with different exopolysaccharide concentrations, adjusted in their pH and the osmotic balance, show that the bacterial suspension containing 3 g.l^{-1} of polysaccharide, had a favorable effect because on the 6 month of age, the cell concentration was in the order of the 5-9x10^8 cell.ml^{-1} keeping the cell's symbiotic properties.

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SELECTION OF AN EFFICIENT AND COMPETITIVE RHIZOBIA INOCULANT FOR COWPEA (*VIGNA UNGUICULATA* L. WALP) CULTIVATED IN THE BRAZILIAN SEMI-ARID REGION

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Cowpea is widely cultivated in the semi-arid region of Brazil, it shows tolerance to water and temperature stress and it is used for human consumption as an important protein source. It is nodulated by a wide range of rhizobial strains, forming associations where the atmospheric N is reduced to ammonium, which is then assimilated by plant tissues, providing better growth and improving grain production. The maximum grain production in legume plants growing under Biological Nitrogen Fixation (BFN) is dependent on the efficiency of the nitrogenase activity and the remobilization of the fixed nitrogen to the pods (Neves, Hungria 1987). The objective of this study was to select an efficient rhizobial strain, under greenhouse conditions, to be field-tested in a further step, as an inoculant for this crop.

The strains were isolated by Martins et al. (1997) from nodules of cowpea cultivated in soil samples from the semi-arid region. The experiment was conducted as a randomised complete block design with 5 repetitions and 5 harvests. The inoculation was performed 3 days after germination (DAG), using 1mL of inoculant per plant. N-free nutritive solution was used once a week. The controls were a nitrogen treatment (150 mg of $N.plant^{-1}$), a non-inoculated treatment and an inoculated treatment with the type strain recommended for cowpea (BR 2001). The harvests were performed at 15, 30, 55, 60 and 75 DAG. Nodules were removed from the roots, counted and weighted. Afterwards, nodules and plants were dried in a forced-draft oven at 70° C and weighted. The total-N analyses were carried out using the semi-micro Kjeldhal's method described by Liao (1981) followed by distillation and titration according to Bremner, Edwards (1965).

A positive correlation was observed between accumulated dry matter and total-N in cowpea inoculated with 10 rhizobial strains, indicating that there is no difference in the remobilization ratio promoted by the rhizobial strains studied. Nodules dry weight also correlated positively with total-N as a consequence of the nodular efficiency being similar among the isolates. Strain 21A9 showed an increase in nodule dry weight when compared with the other isolates. Furthermore, this strain displayed the highest nodule mass at 75 DAG which may be an interesting characteristic for an inoculant strain. Except for strains 8S6, 7S31 and 9A1 the others strains showed similar levels of N accumulation, and might be tested under field conditions to select an efficient rhizobial inoculant for this crop.

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RHIZOBIA STRAINS FROM BRAZILIAN SEMI-ARID REGION ISOLATED FROM ROOT NODULES OF *ARACHIS HIPOGEAE, AESCHYNOMENE* AND *STYLOSANTHES*

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Species of tropical leguminosae are generally nodulated by a large range of rhizobia, therefore BNF contributions in the tropical regions tends to be widely spread. On the other hand, successful introduction of an efficient inoculant is difficult since it has to be more competitive than the indigenous strains. To understand the diversity of the native rhizobia population is crucial to carry on ecological studies, since some characteristics may be related to edapho-climatic conditions on the selection of efficient inoculants for crops of economic interest. The objective of the present study was to select a strain with a more specific host range than the common tropical rhizobia which is used as inoculant for *Arachis hypogaea* (peanut). Besides 10 *Arachis* cultivars, two other genera of the Aeschynomene tribe, will be used as scavanger for rhizobia: *Stylosanthes* (2 species) and *Aeschynomene* (3 species). The plants were cultivated in 7 soil samples from the Brazilian North-East Region. Nodule isolates were characterized according to morpho-physiological characteristics: growth colony rate, colony size and appearance (color, elevation, transparency, shape), change of pH and mucous production. The isolates were clustered using numerical taxonomy analysis, using either single matching (SM) or modified Bray-Curts indices (Ribeiro, 1999) for the similarity matrix and UPGMA for clustering. It has also been worked out the following species richness indices: Margalef and Menhinick. Rhizobia from the native population of the caatinga soil showed a large morphological diversity (Margalef index: 6.5), followed by soil 4 (5.3) and soil 3 (5.0). The lowest indices were displayed in soil 6 and soil 7 (1.9 for both). Most of the isolates (85%) presented fast growth in culture media (YM), but they do not have the typical characteristics described in the Bergey's Manual for rhizobia. Some rhizobial groups showed high specificity in relation to the host plant and were denominated "rare". The cluster analysis of the plant hosts shows similarity between them according to the nodulation capacity with the different rhizobia groups. Three main groups were detected at a 60% similarity level using the Bray-Curts index.

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EFFECTS OF CONCENTRATIONS AND SOURCES OF MOLYBDENUM ON THE SURVIVAL OF *BRADYRHIZOBIUM* STRAINS

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1. Introduction

Molybdenum (Mo) is a component of nitrogenase and thus an essential nutrient for soyabean nodulation. In Brazil, deficiency of this micronutrient has been reported and therefore it has been recommended for application together with the inoculants. However, studies have shown that Mo application on the seeds can reduce *Bradyrhizobium* survival, nodulation and BNF efficiency (Tong and Sadowsky, 1994). This study then aimed to verify the effects of Mo levels and sources on *Bradyrhizobium* survival, nodulation and nitrogen fixation.

2. Material and Methods

The survival of three *B. elkanii* (SEMIA 587, SEMIA 5019 and SEMIA 5079) and one *B. japonicum* (SEMIA 5080) strains was investigated in liquid defined media (Wood and Cooper, 1987), with several concentrations of four Mo sources (sodium molybdate, ammonium molybdate, molybdic acid and molybdenum trioxide). These strains are officially recommended for the use in commercial inoculants in Brazil. The effects of Mo application on the seeds on the bacteria survival were studied in glass house experiments, where the Mo concentrations and sources previously mentioned were compared to commercial products. Bacteria survival, nodulation and N_2 fixation were evaluated.

3. Results

Growth of the four strains of *Bradyrhizobium* varied with the Mo sources and concentrations. The best levels of Mo to the strains were between 0.0125 and 0.05μM of Mo. Sodium molybdate and ammonium molybdate were the best sources of Mo and growth on trioxide of Mo was observed at a maximum concentration of 0.8μM of Mo. *B. japonicum* strain SEMIA 5080 tolerated the highest levels of Mo. The application of Mo on the seeds, together with the inoculant, reduced *Bradyrhizobium* survival, nodulation and N_2 fixation efficiency. Both the non-commercial and commercial sources of Mo showed similar results on the survival of *Bradyrhizobium,* but some sources of Mo were more deleterious to nodulation and N_2 fixation rates.

4. Conclusions

Bradyrhizobium strains differ in relation to the tolerance to Mo concentrations and sources. The application of Mo on the seeds with the inoculant decreases *Bradyrhizobium* survival and often nodulation and N_2 fixation rates.

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DIVERSITY OF *NOD, NIF, DCT,* AND *MOC* GENES AND THEIR RELATION WITH AN EFFECTIVE SYMBIOSIS

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The effectiveness of the symbiosis established between rhizobia and legumes results of a variety of biotic and abiotic conditions. For example, symbiotic gene was correlated with symbiotic effectiveness in *Bradyrhizobium* (Urtz and Elkan, 1996). It has also been reported that genes involved in physiology are important for an effective symbiosis. Batista et *al.* (1992) reported **Dct**$^-$ mutants of *Sinorhizobium meliloti* form ineffective nodules. In this study, RFLP analysis was used to examine the correlation between loci polymorphism and effective symbiosis. Five gene probes were chosen to detect changes in the genes responsible for : the synthesis of nitrogenase (*nifH* and *nifA*); nodule development (*nodA*)*;* transport of **C_4-dicarboxylate** (*dctA*) and the degradation of rhizopine (*moc*A), which was hypothesized confers a competitive advantage upon the bacterial strains harboring it (Rossbach et al. 1995).

Total DNA's of 24 rhizobia (19 *S. meliloti,* 3 *R.* type Oregon strains and one *R. tropici*) were isolated and digested with *Eco*RI and *Bam*Hl. DNA probes were synthesized from Sm 2011 by PCR (Haukka et *al.* 1998) and labeled using biotinylated random primers. Southern blot, hybridization and chemiluminescent detection were carried out using standard protocols. Temperatures for hybridization procedure were : 55°C for *nif, dct,* and *moc* probes and 68°C for *nod* probe. Symbiotic effectiveness was determined by measuring shoot dry-matter of alfalfa plants grown in Jensen-agar medium.

We did not reveal any hybridization pattern with digested DNA from *R.* type Oregon strain CE20. OR191 and CE26 strains hybridized with *nod*A and *nif*H probes respectively. Any R. type Oregon strains hybridized with the regulatory gene *nif*A of Sm. These results could be expected since these strains induce ineffective nodules in alfalfa. All Sm isolates, except one, had identical or nearly identical RFLP pattern when *nif* H gene probe was used. However, when *nif*A probe was used, 5 different pattern (DNA restricted with *Eco*RI) were observed on 19 strains, suggesting notably diversity among *nif* A gene. No polymorphism among *nod*A gene of Sm isolates were revealed on DNA digested with both restriction enzymes. The group of 23 strains were analyzed by RFLP employing a PCR fragment containing part of Sm L5-30 *moc* A as probe. Only seven strains revealed an hybridization pattern, suggesting that the gene responsible to catabolize rhizopine are not extensively present in this rhizobia population. Results obtained by competitiveness studies indicated that some of those seven strains referred above, were more competitive. A possible correlation between the presence of a RFLP pattern with *moc* A and the ability to compete should be confirmed. All the restricted DNAs hybridized with *dct*A probe. The RFLP pattern obtained from Sm strains showed no diversity between them. However, *R.* type Oregon strains revealed a distinctive pattern compared with Sm isolates. The future of this work will be focused in the establishment of a more precise correlation between the presence of molecular markers obtained with mocA gene and the competitive ability of *Sinorhizobium* strains.

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ACETOBACTER DIAZOTROPHICUS EXTRACELLULAR PROTEINS

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Several proteins were detected in the concentrated cell-culture supernatant of *A. diazotrophicus* Pal 3 grown in minimal medium containing sucrose (1%) and ampicillin (50 ug/ml) at pH 5. The protein present in the highest concentration had a molecular mass of 45 kDa. The N-terminal sequence of this protein was determined and the best homology scores obtained for the 19 aminoacid sequence corresponded to the protein flagellin from several microorganisms.

An extracellular polymer synthesizing activity (levansucrase) was also detected in the supernatant. Activity stains of SDS-polyacrylamide gels with different polyacrylamide (7.5%, 10%, 10-15%) and SDS (0.1%, 0.55%) concentrations were performed. Substrate specificity and molecular mass were investigated. Two activity bands with apparent molecular masses of 78 and 40 kDa, having the same substrate specificity, were detected in the cell culture supernatant when the SDS concentration was 0.1%, however only one activity band (40 kDa) was detected in a partially purified fraction and in the cell culture supernatant when the SDS concentration was raised to 0.55%. Polymer formation was visualized as a white band after incubation of gels with different substrates. Activity was detected when sucrose (Glu-Fru), raffmose (Gal-Glu-Fru) and stachyose(Gal-Gal-Glu-Fru) were used as substrates but not with melezitose (Glu-Fru-Glu), indicating that the polymer is formed only with substrates that contain fructose as the terminal residue. The ratio of glucose/fructose released from sucrose by this activity was determined. Levansucrase is usually measured by the release of glucose from sucrose.

A. diazotrophicus levansucrase had a high activity with raffinose as substrate. This allowed the development of a better assay for this enzyme based on the enzymatic measurement of fructose released from raffinose. Pal3 levansucrase gene was PCR-amplified, sequenced and cloned.
A 451 bp fragment of 16SrDNA from strain Pal 3 contained the signature base pairs of *Acetobacter diazotrophicus* strain PAl 5. The diversity of four strains (PAl 5, Ppe4, PR20 and Pal 3) of *Acetobacter diazotrophicus* was studied by rep-PCR genomic fingerprinting using primers designed to anneal to repetitive Box, Eric and Rep elements. Strains PAl 5 and PR20 were most similar and clustered together while strains PPe4 and Pal 3 clustered independently.

MICROBIOLOGICAL RESOURCES CENTER - MIRCEN PORTO ALEGRE.

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The MIRCEN, established in 1978 by an agreement between the Brazilian Government and UNESCO has involved efforts of two institutions, Secretaria de Ciência e Tecnologia/ FEPAGRO and UFRGS/Faculdade de Agronomia - Dept°. de Solos, focusing on the dissemination of technologies in microbiology applied to agriculture and biotechnology. During the period of 21 years, the MIRCEN has trained more than 400 microbiologists, biologists, and agronomists from 20 countries of Latin America, Caribe, and Afrika, through short courses (2-4 weeks) and training periods (3-6 months) related to technologies in biological nitrogen fixation (rizobia/leguminous), biodegradation/ biodeterioration, and food microbiology. The Center maintains a colection with more than 1200 strains of *Rhizobiwn* and *Bradyrhizobium,* located at FEPAGRO, for distribution to research institutions and inoculant industries. It is also responsible for the quality control of imported inoculants or produced by national industry. The staff has attended industry and research institutions concerning to training courses, research projects, and seminars.

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SELECTION OF EFFICIENT AND COMPETITIVE STRAINS OF *BRADYRHIZOBIUM* FOR THE SOYBEAN CROP IN BRAZIL

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1. Introduction

Soybean was introduced in Brazil in the last century, but large scale commercial crop production and inoculation only began in the early 1960s. A search for efficient and competitive strains still takes place, since the bacteria have to meet the increased N demand of more productive cultivars. A program was established consisting of searching, under laboratory and greenhouse conditions, for variant colonies of a single strain for the parameters of higher N_2 fixation capacity and competitiveness.

2. Material and Methods

Strain CB 1809 (=SEMIA 586, =USDA 136b, =TAL 379) was used as the parental genotype. CB 1809 was introduced from Australia to Brazil in 1966, and was classified as very efficient in terms of nitrogen fixation and commercially recommended in 1977, but showed low competitiveness and was unable to nodulate IAC-2, an important commercial cultivar of the 1970s. Therefore the main objective of this study was to obtain variant strains of CB 1809 with higher competitiveness and adapted to the Brazilian soil conditions. Several individual colonies from a pure culture were tested in Leonard jars for competitiveness, with nodule occupancy evaluated by serology. Promising strains were used in field experiments. Bacteria were reisolated from soybean nodules of the inoculated treatments characterized by the best symbiotic performance (nodulation) and yield. Eight isolates from serogroup CB 1809 were selected and used in this study: CPAC 390, CPAC 392, CPAC 393, CPAC 394, CPAC 402, CPAC 403, CPAC 404 and CPAC 405.

3. Results

In relation to the colony morphology, the main difference between parental CB 1809 and the variant strains belonging to this serogroup was that the variant strains produced more mucus. Protein and lipopolysaccharide profiles of variant strains belonging to this serogroup were similar to that of the parental CB 1809. However, in relation to the Nod factors profile, several variant strains differed from parental CB 1809, lacking a band when induced by genistein. Using ERIC and REP-PCR genomic fingerprintings, a very limited level of polymorphism, especially in the region between 600 and 1,000 bp, was detected between CB 1809 and the variant strains. The highest rate of N_2 fixation resulted from inoculation with strain CPAC 390, which allowed an increase of 58% of the N accumulated in soybean shoots in relation to the plants inoculated with the parental CB 1809. These increases were of 58%, 130% and 108% in relation to three other strains officially recommended for the soybean crop, SEMIA 5079 (=CPAC 15), SEMIA 5019 (=29w) and SEMIA 587, respectively. The variant strains showed higher nodule occupancy than the respective parental genotype CB 1809, increasing nodule occupancy in up to 132% (CPAC 390 and CPAC 403).

4. Conclusion

Variant strains showing higher capacity of N_2 fixation and competitiveness can be obtained under laboratory conditions, searching for individual colonies with specific properties.

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SELECTION OF *RHIZOBIUM* STRAINS FOR THE COMMON BEAN CROP

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1. Introduction

Common bean (*Phaseolus vulgaris* L.) is widely cultivated in the Central and South Americas and in Africa, but a low availability of soil N in those areas contributes decisively to the low yield frequently observed. Biological nitrogen fixation therefore plays a key role and can contribute to increase yields, but the process meets several limitations, as poor nodulation, low efficiency of strains and lack of response to field inoculation. However, programs aimed to find efficient and adapted strains have not been consistently conducted. This work reports the results obtained with three strains isolated from the State of Paraná, Brazil.

2. Material and Methods

More than 400 isolates were obtained from 15 soils of the State of Paraná. The isolates were tested under greenhouse conditions for N_2 fixation capacity and three promising strains were identified and named as PRF 35, PRF 54 and PRF 81. Those strains also showed higher competitiveness and, as they were able to nodulate both bean and *Leucaena* plants and could grow *in vitro* at temperatures higher than 37°C, they were classified as *Rhizobium tropici.* Comparisons were made with bean rhizobia type strains: *R. leguminosarum* bv. phaseoli USDA 2671, *R. etli* CNF 42, *R. tropici* type IIA CFN 299 and *R. tropici* type IIB CIAT 899.

3. Results

The three strains from Paraná State and CIAT 899 were tolerant to high temperature (40°C) and acidity (pH 4.0) *in vitro.* They were also able to nodulate beans and fix nitrogen at high temperatures ($37°C\ 8h^{-1}\ day^{-1}$). The strains showed unique profiles of proteins, lipopolysaccharides and DNA amplified by RAPD or ERIC and REP-PCR. The analysis of the PCR products showed that the strains were genetically closer to *R. tropici.* Nod factor profiles were also similar to those of *R. tropici* types IIA and IIB and different from that of *R. etli.* However, mixed characteristics of both types were verified for other parameters, e.g., colony morphology was similar to type IIA, the tolerance to temperature and acidity was similar to type IIB and the Y1-Y2 PCR product had the same molecular weight as type IIB. The analysis of 17 fatty acids classified two strains as type IIA (PRF 35 and PRF 54) and one as type IIB (PRF 81). At the field, inoculation with PRF 81 resulted in statistically significant increases in nodulation, nodule occupancy, total N accumulated in tissues and yield. The benefits of inoculation with PRF 81 were confirmed in several other field sites and the strain started to be officially recommended for the bean crop since 1999. The new designation for PRF 81, according to the Brazilian germplasm bank, is SEMIA 4080. Now other promising isolates from the Central Region of Brazil, the Cerrados, are being tested.

4. Conclusion

The good symbiotic performance of strain PRF 81 encourages the identification of new competitive, efficient and genetically stable *Rhizobium tropici* strains for the bean crop in tropical regions.

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SYMBIOTIC PERFORMANCE OF SOYBEAN SINORHIZOBIA STRAINS ISOLATED IN BRAZIL

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1. Introduction

Soybean (*Glycine max* (L.) Merrill) is an exotic plant introduced in Brazil one hundred years ago. Brazilian soils are originally free of *Bradyrhizobium japonicum* and *B. elkanii* and there are also no reports of isolation of *Sinorhizobium fredii.* A survey was carried out using, as trapper plants, one modern and six non-bred soybean genotypes, inoculated with soils from 22 Brazilian field sites, including undisturbed or soybean traditionally cultivated areas. Thirty fast growing strains able to establish an efficient symbiosis with both non-bred and modern soybean genotypes were isolated. The strains resemble *Sinorhizobium fredii,* although showing unique properties (Mateos et al., in this Proceedings). Here, we have investigated the symbiotic performance of these 30 strains when compared with other *S. fredii* and *B. japonicum/B. elkanii* strains.

2. Material and Methods

Nitrogen fixation capacity was evaluated in soybean plants (cv. BR-16) grown in Leonard jars and single inoculated with each strain (10^9 cells ml^{-1}, 1 ml $seed^1$). For evaluation of nodule occupancy, soybean seeds (cvs. BR-16 and Davis) received a mixed inoculum of each strain and of *B. elkanii* SEMIA 5019 (10^9 cells ml^{-1}, 1:1, v: v, 1 ml $seed^{-1}$). For the evaluation of effects of pH on nodulation and nitrogen fixation, samples of an oxisol with pH 5.1 received lime for four months until they reached pHs 6.8 and 8.0. Pots of 5 kg capacity were filled with 4 kg of soil (with an established population of 10^4 cells of *Bradyrhizobium* g^{-1} soil), with four replicates per treatment.

3. Results

When single inoculated under sterile conditions, several Brazilian isolates fixed as much nitrogen as the *B. japonicum/B. elkanii* strains officially recommended for commercial inoculants in Brazil. Nitrogen fixation rates of 23 of the 30 Brazilian sinorhizobia were also higher than those observed with soybean inoculated with *S. fredii* type strains USDA 205 and CCBAU 114. However, sinorhizobia were poor competitors when co-inoculated with *B. elkanii. S. fredii* USDA 205 and CCBAU 114 were also not able to compete against *B. elkanii* SEMIA 5019. Brazilian strains were isolated from soils with a pH ranging from 3.04 to 5.09. However, in pots containing non-sterile soil and pH 5.5 or pH 6.2, nodule occupancy by the sinorhizobia was usually very low, increasing considerably with massive liming (pH raised to 8.0). The Brazilian strains could be grouped into eight Nod factors and 11 lipopolysaccharide profiles, but none of the groupings was related to N_2 fixation capacity or competitiveness. Other characteristics of the 30 Brazilian sinorhizobia strains, such as time of generation, growth in different C and N sources, among others, also did not correlate with N_2 fixation capacity or competitiveness.

4. Conclusions

As most Brazilian soils are acidic (pH below 6.0), the use of sinorhizobia strains in inoculants for the soybean crop is not recommended at this moment, because they are poor competitors at low pH.

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INOCULATION EFFICIENCY IN *INGA EDULIS* WITH DIFFERENT *BRADYRHIZOBIUM* STRAINS ON OXISOL FROM AMAZONIA

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This glasshouse study aims to evaluate the inoculation efficiency in *Inga edulis* with 23 different *Bradyrhizobium* strains (from INPA's Rhizobia Collection) on a Oxisol (Latossolo Amarelo) six months after slash-and-burn, traditional system of soil preparation in the Amazonia. The inoculation was realised at five days and the evaluation at 120 days after sowing. The following parameters were measured in *I. edulis* seedlings: weight of aboveground dry matter , number and weight of nodules and foliar nitrogen concentration. The number and weight of nodules and the concentration of nitrogen were significantly different between inoculation with different strains. However, the treatments did not have significant effects on growth of *I. edulis.* The inoculation with strains 529-C9B, 529-C6B, 529-C6B and 529-B6A had the best performance. These strains showed good potential to use in inoculum production for *I. edulis.* No nodulation was found in treatments without inoculation, indicating that it is necessary to use an inoculation technique under the described conditions of land use.

NODULATION COMPETITIVENESS OF *BRADYRHIZOBIUM* STRAINS HARBOURING AN ADDITIONAL HETEROLOGOUS *nifA* GENE

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A major barrier to the use of nitrogen-fixing inocula for the enhancement of legume productivity is the inability of commercially available strains to compete with indigenous rhizobia for nodule formation. Various physiological attributes and some genes have been shown to be involved in the competitive ability of rhizobia strains (Triplett, Sadowsky, 1992). One strategy to improve the competitiveness is to introduce genes that give the recipient bacteria an advantage over the established strains (Soto et al., 1993; Chun, Stacey, 1994; Robleto et al., 1998). Another strategy would be the introduction or alteration of the regulatory genes. The rational of this approach is that several genes can be activated before the infection of the plant.

The role of NifA in rhizobia is not limited to the regulation of the nitrogenase and accessory genes but includes additional functions which are more generally related to the symbiosis (Fischer, 1994). Surprisingly, mutations in *nifA* affect the nodulation process in rhizobia and in *B. japonicum* the wild-type nodulation is restored by heterologous complementation with *nifA* from *Klebsiella pneumoniae.* To test the effect of *nifA* on nodulation competitiveness of *Bradyrhizobium,* the plasmids pCK3 and pML3A carrying the constitutive *nifA* genes from *Klebsiella pneumoniae* and *Azospirillum brasilense,* respectively, were transferred to the *B. japonicum* CPAC 7 and *B. elkanii* 29W strains. The root nodule occupancy of soybean and plasmid stability were determined under greenhouse conditions. In both cases the competitiveness was enhanced statistically (χ^2 test, $p < 0.05$) despite the low persistence of the vector in these strains in the nodule and when grown in PSY medium without selection pressure. Strain CPAC 7 harbouring plasmid pML3A occupied 75% of the nodules even when co-inoculated with the wild-type strain at a 1:10 ratio.

To achieve a stable expression of the heterologous *nifA* gene a recombinant plasmid capable of site-directed chromosomal integration is been constructed. A non-essential DNA segment carrying a repeated sequence (like IS) was isolated by PCR amplification from *B. japonicum* strain CPAC 7, cloned in pTZ19R and sequenced. Restriction sites were introduced in this sequence to clone the *nifA-uidA-aph* genes. A suicide plasmid containing the *Klebsiella pneumoniae nifA* gene, transcribed from a strong promotor (*ptac*), was constructed and transferred to *Bradyrhizobium* strains. Mutants containing the integrated *nifA* gene will be analysed for nodulation competitiveness. It is expected that utilization of a chromosomal integration vector will allow more conclusive studies of the involvement of *nifA* and other genes in *Bradyrhizobium* nodulation competitiveness.

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A *RHIZOBIUM ETLI* CFN42 MULTIRESISTANCE EXTRUSION PUMP INDUCED BY BEAN-ROOT EXUDATE

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We found an ORF in *R. etli* strain CFN42 inducible by *Phaseolus vulgaris* bean-root exudate and by flavonoids. The Tn*5*-B30 mutant has reduced nodulation capacity on *P. vulgaris,* accumulates genistein, and has enhanced sensitivity to root exudates, some flavonoids, and salicylic acid compared to the wild type strain. Nucleotide sequence analysis showed that this ORF is predicted to encode a 41.8 kDa protein that has similarity with members of MFP family, designated *rmr*A (rhizobium multiresistance gene A). *rmr*A is probably organized as an operon together with a downstream ORF, rmrB, which encodes a predicted 56.1 kDa protein with properties typical of members of the MF superfamily. A third ORF placed upstream of *rmrA,* designated *rmr*R*,* specifies a putative 13.0 kDa polypeptide that has similarity to Tet/Acr family (transcriptional regulators) and contains a helix-turn-helix motif. These genes are located on the LPS plasmid (pb, 200 kb).

We propose that *rmr*AB encodes a membranal export system spanning the two membranes that prevents the accumulation inside the cell of toxic compounds produced by bean-roots and that *rmr*R encodes a regulatory component of the *rmr*AB operon. This transport system seems to confer to *R. etli* an advantage for nodulating bean plants.

DIVERSITY OF RHIZOBIA ISOLATED FROM *PTEROCARPUS ERINACEUS* (POIR.) AND *PTEROCARPUS LUCENS* (LEPR.) AND NITROGEN FIXING POTENTIAL OF SYMBIOSIS

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Pterocarpus erinaceus and *P. lucens* are two important nitrogen fixing trees in tropical arid and semi-arid zones.

Characterisation of rhizobia isolated from root nodules of the two *Pterocarpus* species was done using SDS-PAGE proteins analysis and full-length 16S rDNA sequence comparison. Numerical data analysis showed that these strains included fast- and slow-growing rhizobia. The fast-growing rhizobia are closely related to *Rhizobium leguminosarum, Rhizobium tropici* or *Mesorhizobium plurifarium* species. Slow-growing rhizobia are closely related to *Bradyrhizobium japonicum, Bradyrhizobium* sp.*lupinus* or *Bradyrhizobium elkanii.*

Measurements of N_2 fixation in *Pterocarpus erinaceus* and *P. Lucens* were done using the ^{15}N direct isotope and A-value methods. A non fixing plant *Dialium guineensis* served as a reference tree. Results showed that for each of the plant species the %Ndfa was in the range of 35%, 60% of the total N contained in plants was derived from soil and only 5% was derived from fertiliser.

COMPETITION BETWEEN *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS NGR 234 AND CP 283 FOR NODULATION IN SIRATRO INVESTIGATED WITH GUS REPORTER GENE

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1. Introduction

Siratro (*Macroptilium atropurpureum*)(DC.) is a forage legume, which forms effective nodules by various *Rhizobium* or *Bradyrhizobium* strains. The use of GUS transposon as a marker for rhizobial competition studies, is very easy, reliable and sensitive. In the present investigation we described a very interesting and new report on competitiveness of *Rhizobium* strain NG 234 and *Bradyrhizobium* strain CP 283 for nodulation in siratro using GUS reporter gene.

2. Material and Methods

We constructed several gusA-marked mutant strains of NGR 234 and CP 283. Out of the constructed gusA-marked strains, only two were used in this study. The gusA-marked stran of NGR 234 and CP 283, which were used in this study are indicated as NGR 234 a-3 and CP 283 N-2, respectively. The seeds of siratro (*Macroptilium atropurpureum*) were surface sterilized and were germinated on sterile vermiculite in jars. One day after transplanting, the seedlings were inoculated with the different strains and concentrations. For the single inoculation, the seedlings were inoculated separately with 1 ml cell suspension of NGR 234 and Cp 283 and their gusA marked strains NGR 234 a-3 and CP 283 N-2 at 10^7 and 10^3 cells plant^{-1}. For the co-inoculation, cells of both the *Rhizobium* strain NGR 234 and CP 283 and the gusA marked *Bradyrhizobium* strain CP 283 N-2 were suspended in cell ratio of 1:1 and at a cell density 10^7 cells ml^{-1}. Similarly, cells of the *Bradyrhizobium* strain CP 283 and gusA marked *Rhizobium* strain NGR 234 a-3 were mixed in a ratio of 1:1 and at a cell density 10^7 cells ml^{-1}. Half of the mixed suspensions was then diluted with sterile water to 10^3 cells ml^{-1}. One milliliter of the above mixed suspensions having 10^7 and 10^3 cells ml^{-1} were inoculated separately to each plant. Nodule formation was monitored every day and nodule number and fresh weight were recorded at 7, 10, 14, 21, 28 and 35 days after inoculation (DAI). The plants were harvested at 10, 14, 21 and 28 DAÍ for GUS assay the specimen were finally observed by bright-field microscopy with an Olympus light microscope.

3. Results and Discussion

The nodules induced by NGR 234 and CP 283 were not stained blue when treated with X-gluc., but when induced with gusA marked strains NGR 234 a-3 and CP 283 N-2 stained blue, indicating that these nodules were induced by gusA marked *Bradyrhizobium* strain CP 283 N-2. Thus, in these co-inoculated plants 21-24 % of the nodules were occupied by *Bradyrhizobium* strain CP 283 and 76-79 % by the *Rhizobium* strain NGR 234. When these strains were co-inoculated, the *Rhizobium* strain NGR 234 was found more competitive than the *Bradyrhizobium* strain CP 283.

NEW RECORDS OF TROPICAL LEGUMES NODULATION

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Most plants of the legume family establish a symtiotic relationship with soil bacteria known collectively as "*Rhizobium*" (rhizobia). The symbiosis is manifested by the formation of nodules, specialized organs found in the roots or rarely on stems. In the nodules, the rhizobia transform into bacteroids that are able to reduce N_2 to ammonia that is assimilated by the plant. In this way, legumes can grow in soils low in nitrogen and can even supply some N to accompanying crops or those following in a rotation.

Only a small percentage of the thousands of existing legumes are known as nodulating. This is particularly important in tropical America, where the largest diversity of species exists. In Puerto Rico, there are more than 200 known species of legumes, but nodulatin is unknown in many cases. We have previously reported the lack of nodules in *Delonix regia* (Schroder, 1984). Our research work is focused on obtaining that information and characterize the rhizobia isoalted in Puerto Rico and the Caribbean.

We have recently found nodules in *Calliandra locoensis* and *Pictetia acueleata.* However, the strains were unable to nodulate *Macroptilium atropurpureum* cv. Siratro, but produce short, deformed roots in some plants.

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SELECTION PROGRAM FOR ROOT NODULE BACTERIA IN MEDITERRANEAN AGRICULTURE IN AUSTRALIA

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The Centre for *Rhizobium* Studies has, as part of its research program, selected new strains of root nodule bacteria for pastures (*Medicago, Trifolium, Ornithopus, Biserruld*) and pulses (*Lens, Vicia*) grown on a mix of soil types from alkaline sandy-loams to infertile acid sands in Mediterranean regions of southern Australia.

A four phase selection process has been used: germplasm collection from the Mediterranean basin; authentication and screening for effectiveness; *in situ* field trials for adaptation to soils; assessment of strain stability for inoculum production.

An essential trait for commercial inocula in Australian agriculture is high effectiveness with a broad range of hosts. Several genera of pulses (*Vicia, Lens, Pisum and Lathyrus*) are serviced with one or two inoculants, and ten *Trifolium* spp. are inoculated with only two strains.

New developments are the selection of strains for pulses being introduced onto marginal soils and perennial herbacious pasture legumes wth a potential for dewatering the landscape in southern Australia.

SECTION XIV

NITROGEN FIXATION IMPERATIVES FOR THE 21ST CENTURY

PROSPECTS FOR ENGINEERING NITROGEN-FIXING PHOTOSYNTHETIC EUKARYOTES

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1. Introduction

The long-term goal of engineering autonomous nitrogen-fixing cereal crops has been considered at many previous international meetings on nitrogen fixation. The overall consensus appears to be that this is a pioneering, ambitious project with many potential barriers to its success, some of which may be insurmountable. However this viewpoint requires frequent re-evaluation in the light of recent developments in biotechnology and the imperative to sustain the ever increasing human population. Notably, recent advances in plant molecular biology and technical developments in cereal transformation have facilitated the introduction of multiple genes into important food crops (Kohli et al, 1998). Therefore molecular 'tailoring' of *nif* genes to allow efficient expression of nitrogenase proteins in cereal plants does not itself present a major problem in the short term. However the expression of nitrogenase in a eukaryotic environment necessitates provision of the appropriate physiology to support the biosynthesis and activity of nitrogenase, in addition to providing a route for assimilation of the nitrogen that is fixed (Dixon et al, 1997). After due consideration of these requirements, one might conclude that the environment provided by a root-nodule-like structure may be the only solution in terms of protecting and supporting nitrogen-fixation in plants. However, if one considers the various ways in which aerobic diazotrophs are able to support nitrogen fixation under oxic conditions, alternative solutions cannot be ruled out. For example, the ability of the cyanobacteria to reconcile photosynthetic oxygen evolution with the oxygen-sensitivity of conventional nitrogenase may provide a paradigm for the engineering of photosynthetic diazotrophic eukaryotes.

2. Cyanobacteria as a Model

The expression of nitrogenase in cyanobacterial heterocysts provides an excellent example of compartmentation and hence protection of the enzyme from photosynthetic oxygen evolution. On the other hand, non-heterocystous cyanobacteria also reconcile photosynthesis and nitrogen fixation by separating both processes on a temporal basis. In *Gleothece* for example, nitrogenase is synthesised during the dark phase supported by ATP and reductant provided by the breakdown of endogenous glucan (Gallon, 1992). When supplies of energy and reducing power become limiting, nitrogenase synthesis ceases and its activity is lost. Glucan reserves are replenished during the light phase to support further nitrogen fixation in the dark. Although this could provide a model for the temporal separation of nitrogen fixation and photosynthesis in transgenic chloroplasts, the levels of ATP and reductant available during the dark period are probably insufficient to support nitrogen fixation in higher plants. Nevertheless, when exposed to continuous illumination, non-heterocystous cyanobacteria remain competent to fix nitrogen and this phenomenon has yet to be explained.

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3. Lower Plants as a Model

It is a commonly accepted evolutionary hypothesis that plastids evolved from photosynthetic prokaryotes, probably cyanobacteria. These ancestors may have also been diazotrophs since DNA sequencing of lower plant chloroplast genomes has revealed three genes, designated *chlL, chlN* and *chlB* which may be structurally and functionally related to nitrogenase component proteins. In particular, the structural subunit of nitrogenase Fe protein (NIFH) encoded by *nifH,* exhibits 32-35% identity with the putative "chlorophyll iron protein" (CHLL) encoded by *chlL* (Burke et al, 1993, Fujita et al, 1992, Suzuki and Bauer, 1992). Cysteine residues required for liganding the Fe_4-S_4 cluster in Fe protein are conserved in CHLL, raising the possibility that it may also be an oxygen sensitive protein. The sequence identity between these proteins is maintained over their entire length and in addition to the environment surrounding the cysteine residues, the ATP binding pocket is also conserved. The *chl* genes are required for the dark-dependent pathway of chlorophyll biosynthesis in which chlorophyll a and b can be synthesised in complete darkness. Since *M* mutations prevent the conversion of protochlorophyllide to chlorophyllide in the dark, leading to a yellow-in-the-dark' phenotype, it has been suggested that the three *chl* gene products comprise the structural subunits of a light-independent protochlorophyllide reductase (DPOR) (Fujita, 1996). DPOR may have evolved from an ancestral nitrogenase to perform chlorophyll biosynthesis under anaerobic conditions, in contrast to light-dependent protochlorophyllide reductase (LPOR) which subsequently evolved to permit porphyrin biosynthesis in the presence of oxygen and light. Two interesting questions emerge from this hypothesis. Firstly, if DPOR is indeed an oxygen-sensitive enzyme, is the chloroplast environment sufficiently anoxic during the dark period to prevent oxygen damage to nitrogenase? Secondly, if CHLL, by analogy with nitrogenase Fe protein is a reductase component, which consumes ATP and reducing power to donate electrons for substrate reduction by DPOR, is there sufficient energy and reductant available in the dark period to support nitrogenase activity?

We have explored potential structural and functional relationships between nitrogenase Fe protein and CHLL using the photosynthetic eukaryote *Chlamydomonas reinhardtii* as a model system. When grown heterotrophically in the dark on acetate-containing medium this organism produces green colonies due to the action of DPOR. Mutations in the plastid-encoded *chl* genes give rise to a 'yellow-in the dark phenotype' since chlorophyll biosynthesis is prevented under these conditions. Chloroplast transformation is well established in this organism and we have exploited this to make targeted insertions in the plastid genome in which the coding sequence of *chlL* is precisely replaced, either by the *Klebsiella pneumoniae nifH* coding sequence or by the marker gene *uidA* which encodes *β*-glucoronidase. To do this we inserted an antibiotic resistance cassette into a region closely linked to the gene of interest and then performed a second transformation to replace the cassette and introduce the foreign gene by homologous recombination (Fig 1).

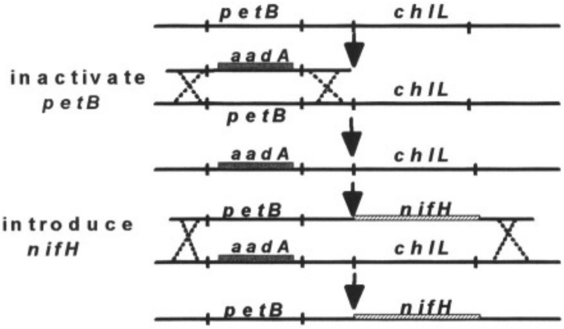

Fig 1 Strategy for the introduction of foreign genes into the plastid genome of *C.reinhardtii* by homologous recombination

In the initial transformation *petB,* a photosynthetic gene closely linked to *chlL,* was inactivated through insertion of a spectinomycin resistance gene (*aadA*). ***PetB***$^{-}$ mutants require a reduced carbon source such as acetate for growth since they are unable to grow photoautotrophically. In a second transformation we introduced a DNA fragment carrying a wild-type copy of the *petB* gene, together with closely linked *nifH* or *uidA* coding sequences flanked by the regulatory regions of *chlL.* Transformants were sought in which homologous recombination had replaced the *aadA* insertion in *petB* with the wild-type *petB* gene and the *nifH* or *uidA* sequences had replaced the coding sequence *of chlL.* Many of the transformants selected for further study had a yellow or partial green phenotype when cultured on acetate medium and incubated in the dark, indicative of replacement of the wild-type *chlL* sequence by foreign DNA sequences. Transformants which carried the expected insertion of foreign DNA and which were homoplasmic for the transgene were identified by Southern hybridisation. Homoplasmic *uidA* transformants were yellow when cultured in the dark on acetate medium, in contrast to the wild-type and the petB::aadA insertion mutant which were green in the dark. In contrast, the homoplasmic *nifH* transformant was partially green in the dark suggesting that it may be able to partially substitute for the function of *chlL.* Pigments synthesised by transformants when grown in the dark were analysed by fluorescence spectroscopy. (Fig 2).

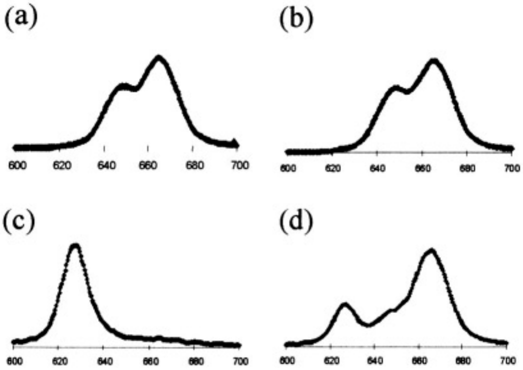

Fig 2 Fluorescence emission spectra of pigments extracted from dark-grown *C.reinhardtii* strains. Chlorophyll a and b exhibit emission maxima at 666 and 648 nm respectively, whereas protochlorophyllide shows an emission maximum at 627nm. (a) wild-type, (b) *petB::aadA* transformant, (c) *chlL::uidA* transformant, (d) *chlL::nifH* transformant

The wild type strain showed the characteristic emission spectrum for chlorophyll a and b in contrast to the *uidA* transformants which accumulated only protochlorophyllide The *nifH* transformant also accumulated protochlorophyllide but the spectrum indicated that a high proportion of the substrate had been converted to chlorophyll. These results show that the *uidA* transformant is deficient in light-independent chlorophyll biosynthesis and the *nifH* transformant is competent to partially restore the capacity for chlorophyll biosynthesis in the dark.

The ability of NIFH to partially complement the function of *chlL* in the dark-dependent chlorophyll biosynthesis pathway supports the notion that CHLL and nitrogenase Fe protein have similar structural and functional properties. Thus CHLL may function in ATP-coupled electron transfer to the other components of the protochlorophyllide reductase encoded by CHLN and CHLB which have sequence similarities with NIFD and NIFK (Fujita, 1996). The ability of Fe protein to partially replace CHLL suggests that oxygen sensitive enzymes may function in plastids when grown in the dark. Furthermore, since *Chlamydomonas* carries out fermentative metabolism under dark-grown conditions, there may be sufficient reductant and energy to support DPOR and by inference, nitrogenase under these conditions. Assuming that a [4Fe-4S] cluster is required for NIFH and

CHLL function in the protochlorophyllide reductase complex, the biosynthesis of this cluster is presumably achieved by ancillary proteins in *Chlamydomonas* which are homologs of *nifU* and *nifS.* The product of a third gene *nifM* is required for activation and stability of the Fe protein in diazotrophs (Peters et al, 1995) and a similar protein may be required for maturation of CHLL in the algal plastid. Since homologues of *nifU, nifS* of *nifM* are not present in the liverwort chloroplast genome, (the only lower plant plastid genome which has been completely sequenced) it is tempting to speculate that their equivalents are encoded in the nuclear genome. Mutations in these genes may be represented among the nuclear mutant 'y' alleles that give 'yellow-in-the-dark' phenotypes in *C. reinhardtii.*

4. Future Prospects

The ability of *nifH* to at least partially substitute for the function of *chlL* in algal cells grown in the dark suggests that the environment of the plastid may be appropriate to support nitrogenase function under these conditions. It may therefore be possible to engineer a diazotrophic derivative of *C. reinhardti* by introducing the remaining *nif* genes required for nitrogenase activity into the plastid, although this will require some engineering to ensure that the coding sequences are flanked by appropriate regulatory sequences. Even if conditions are appropriate for nitrogenase activity in *Chlamydomonas* plastids under dark-grown conditions, where energy and reductant are provided by starch fermentation, this does not necessarily mean that the system can function in higher plants. Firstly, the requirements for nitrogenase biosynthesis are likely to be more complex in a higher plant. Whereas assembly of the Fe protein in *Chlamydomonas* may be facilitated by nuclear equivalents of *nifU, nifS* and *nifM,* the biosynthesis of this protein in higher plant plastids will most likely require the introduction of these genes into the plastid genome. Secondly, the levels of ATP and reductant available during the dark period are probably insufficient to support nitrogen fixation in chloroplasts and there is probably no efficient respiratory system that can remove oxygen under these conditions. It is also questionable whether there is sufficient energy and reductant to support nitrogenase in heterotrophic root plastids.

The possibility of expressing nitrogenase in a leaf environment may confer several advantages if the energy and reducing power generated during photosynthesis in the chloroplast can be harnessed to support nitrogen fixation. The recently discovered oxygen-tolerant nitrogenase from *Streptomyces thermoautotrophicus* may be of considerable benefit in this respect, provided that the plant will tolerate the generation of the superoxide radical required as an electron donor to this enzyme (Ribbe et al., 1997). However, unless alternative electron carriers can be utilised, this novel nitrogenase is only likely to function in the plastid under conditions of photooxidative stress. Introduction of this enzyme into chloroplasts might be beneficial in terms of increased stress-tolerance but nitrogenase activity would be conditional upon stress conditions. Potentially, it might be possible to activate electron transfer to the enzyme by disabling superoxide dismutase, but this may lead to increased levels of hydrogen peroxide, necessitating the engineering of increased levels of peroxide scavenging enzymes such as ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase. Further studies on the mechanisms whereby plants recover from photoxidative stress might be beneficial in terms of establishing appropriate conditions for concurrent nitrogen fixation and photosynthesis. Under conditions of illumination, the chloroplast stroma provides a reducing environment which might allow even conventional nitrogenase Fe protein to reduce oxygen without inactivation. When the concentration of Fe protein is at least in 4-fold molar excess over the concentration of oxygen, the protein is oxidised without loss of activity, a phenomenon known as autoprotection (Thorneley and Ashby,1989). However a major product of this reaction is hydrogen peroxide, so in order to prevent oxidative stress it would probably be necessary to increase the levels of enzymes which remove peroxide as discussed above.

It is a major challenge to attempt to mimic the cyanobacteria and engineer a nitrogen-fixing photosynthetic organelle. From an engineering point of view, the plastid provides a convenient location for the introduction of *nif* genes since chloroplast genes are expressed in a prokaryotic-like fashion, allowing translation of polycistronic messages. However we still have to face the problem of interfacing plastid physiology with the requirements for nitrogenase activity as discussed above. As an alternative strategy, one might consider introducing nitrogenase proteins into mitochondria which may provide a suitable energy-rich, reducing environment to support nitrogenase. However this introduces more complexity since each gene would have to be specifically modified to allow targeting each of the *nif*-encoded proteins into this organelle in the appropriate stoichiometry. Paradoxically, whilst the technology is available now to introduce and express nitrogenase component proteins in plant cells, substantial progress is limited by gaps in our fundamental knowledge of both plant and microbial physiology. For example, there is limited information regarding oxygen concentrations in plant tissues and we still do not have an in-depth understanding of how non-heterocystous cyanobacteria reconcile photosynthesis and nitrogen fixation. Future advances in this pioneering field will undoubtedly be underpinned by fundamental studies on plant physiology in addition to further research on the molecular biology, physiology and biochemistry of nitrogen fixation.

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NITROGEN FIXATION IN RICE: OBJECTIVES AND ACHIEVEMENTS

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1. Introduction

Nitrogen is the nutrient that most frequently limits agricultural production. Global agriculture now relies heavily on petroleum-based nitrogen fertilizers, which are expensive inputs, costing agriculture more than US$ 45 billion per year, and fertilizer is vulnerable to political and economic fluctuations in oil markets. Manufacturing fertilizer for today's global needs requires fossil fuel energy equal to about 100 million t of oil per year. Oil is a nonrenewable resource and its oxidized products pose hazards to human health and to the environment (Bockman 1997). Moreover, fossil fuel reserves are finite and therefore unsustainable in the long term.

Rice needs 1 kg of nitrogen to produce 15–20 kg of grain. Lowland rice in the tropics can use enough naturally available N to produce 2–3 t ha^{-1}. For higher yield, additional N must be applied. Prior to the advent of chemical fertilizers, rice productivity depended almost entirely on biological nitrogen fixation. During the Green Revolution, and since the 1960s, the application of chemical nitrogen fertilizers boosted rice yields by 100–200% to match the demands of the increasing human population. In the next 25 years, we must produce nearly 70% more rice than the 460 million t of today. To enhance rice production from the present 8 t ha^{-1} to 12 t ha^{-1} in 2020 would require an increase in fertilizer application from 220 to 400 kg ha^{-1}. At current levels of N-use efficiency, we will require approximately double the 10 million t of N fertilizer currently used each year for rice production.

2. Need for *in planta* Biological Nitrogen Fixation: Looking Ahead

Rice suffers from a mismatch of its nitrogen demand and the nitrogen supplied as fertilizer, resulting in a 50–70% loss of applied N fertilizer, which eventually contributes to the degradation of the environment by groundwater contamination, nitrous oxide emission, etc. A regulated supply of N is likely to maximize the yield potential of rice, and this could be accomplished through two basic approaches. One is to coordinate the time of N application based on the plant's need. This can partially increase the efficiency of use of applied nitrogen. The other is to increase the ability of the rice plant to fix its own nitrogen (Ladha, Reddy 1995; Ladha et al. 1998). If a BNF system could be assembled in the rice plant, it could amplify nitrogen supply potential because fixed nitrogen would be available to the plant directly with little or no loss. Such a system would give farmers no additional economic burden. This latter approach is a long-term strategy with large public and environmental benefits, while helping resource-poor farmers.

3. A Global Collaborative Initiative

In 1992, the International Rice Research Institute organized a workshop to assess the feasibility of developing nitrogen-fixing capability in rice (Khush, Bennett 1992). The experts attending the meeting identified four major short- and long-term approaches: (1) improving the endophytic associations between rice and nitrogen-fixing bacteria, (2) laying the foundation for the engineering of rice plants capable of forming legume-like symbiosis/nodules with rhizobia, (3) transforming rice with genetic mechanisms to ensure the expression of nitrogenase and protection of nitrgenase from inactivation by oxygen, and (4) refining the processes to enhance N-use efficiency in rice. The

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workshop participants acknowledged the challenge of achieving (endo)symbioses/nodulation and nitrogen fixation in rice through genetic engineering and agreed to work toward that goal (Table 1). Based on the workshop recommendations, IRRI developed a New Frontier Project to coordinate worldwide collaborative efforts among research centers committed to reducing dependency of rice on mineral nitrogen resources (Ladha et al. 1997a). An international BNF working group was also established to review and share research results/materials and to catalyze research. Two BNF working group meetings were held, at IRRI (Philippines) in 1995 and at NIBGE (Pakistan) in 1996 and the proceedings comprising the deliberations of the second working group meeting was published (Ladha et al. 1997b).

Table 1: Universities/research institutions engaged in collaborative/independent research on nitrogen fixation in rice and other cereals

Country		Institution	Research theme with rice[1]/wheat[2]
Autralia	:	Australian National University	Endophyte/rhizobial interaction[1, 2]
		University of Sydney	Associative diazotroph interaction[1, 2]
Belgium	:	Katholieke Universtiet Leuven	Associative diazotroph interaction[1]
Brazil	:	EMBRAPA	Endophyte interaction[1]
China	:	Chines Academy of Agricultural Sciences	Endophyte/rhizobial interaction[1, 2]
		University of Shandong	Endophyte/rhizobial interaction[1, 2]
Egypt	:	Sakha Agricultural Research Station	Rhizobial interaction[1]
France	:	INRA-CNRS, Toulouse	Rhizobial interaction[2]
Germany	:	Max-Plank Institute, Marburg	Endophyte interaction[1]
India	:	Banaras Hindu University	Endophyte interaction[1]
Japan	:	National Institute of Agrobiological Resources	Rice *ENOD* homologues, chitin recognition and defense response[1]
Pakistan	:	NIBGE	Endophyte interaction[1]
Philippines	:	International Rice Research Institute	Endophyte/rhizobial interaction, legume *ENOD* gene expression in rice, rice *ENOD* homologues[1]
		University of the Philippines at Diliman	Endophyte interaction[1]
Switzerland	:	ETH	*ENOD* gene expression in rice[1]
UK	:	John Innes Centre	Expression of *nif* genes in plant cells
		University of Dundee	Endophyte/rhizobial interaction[1]
		University of Nottingham	Rhizobial interaction[1, 2]
USA	:	Michigan State University, East Lansing	Endophyte/rhizobial interaction[1]
		University of Arizona, Tucson	Endophyte interaction[1]
		University of California, Davis	Endophyte/rhizobial interaction[1]
		University of Tennessee, Knoxville	Legume *ENOD* gene expression in rice[1]

EMBRAPA: Empresa Brasileira de Pesquisa Agropecuaria; ETH: Eidgenossische Technische Hochschule Zurich; INRA-CNRS: Institut National de la Recherche Agronomique-Center National de la Recherche Scientifique; NIBGE: National Institute for Biotechnology and Genetic Engineering.

4. Frontier Project on “Assessing Opportunities for Nitrogen Fixation in Rice”: Research Advances (1994-99) and Future Directions

The strategies enabling rice to fix its own nitrogen are complex and of a long-term in nature. Nonetheless, since the initiation of the New Frontier Project “Assessing the Opportunities in Nitrogen Fixation in Rice” in 1994, the worldwide collaborative efforts led to a remarkable progress in the research areas of rice-endophytic diazotroph associations and determination of the genetic predisposition of rice for symbiosis with rhizobia. In addition, concerted research efforts are also underway to elucidate the genetic, biochemical and physiological requirements for the assembly and function of nitrogenase enzyme complex in plant cells (Dixon et al. 1999). In the next five to ten years, we envisage even greater progress towards realizing the goal.

5. Endophytic diazotroph associations

Diazotrophs such as *Acetobacter diazotrophicus* and *Herbaspirillum* spp. grow endophytically in the stems and leaves of sugarcane. Evidence shows that *A. diazotrophicus* is the main contributor of endophytic BNF, which, according to nitrogen-balance studies, was as high as 150 kg N ha^{-1} yr^{-1} in sugarcane (Dobereiner et al. 1993). Another nitrogen-fixing endophyte of considerable interest is *Azoarcus*. This diazotroph inhabits the roots of Kallar grass (*Leptochloa fusca*), which yields 20–40 t of hay ha^{-1} yr^{-1} without the addition of any N fertilizer in saline-sodic, alkaline soils having low fertility (Sandhu et al. 1981). These investigations suggest that endophytic diazotrophs have a considerable potential to contribute to the productivity of nonlegumes, including rice. With a methodical approach, scientists at IRRI and collaborating institutions are pursuing the following research initiatives to explore and evolve strategies to improve rice-endophytic diazotroph associations:

- To identify specific and predominant endophytic diazotrophs from rice.
- To determine the mode of invasion and extent of colonization by endophytic diazotrophs.
- To assess the contribution of endophytic diazotrophs to rice growth and yield through nitrogen fixation and growth promotion.

First, the method for surface sterilization of rice tissues was standardized to enable the proper enumeration and isolation of putative endophytes (Barraquio et al. 1997; Stoltzfus et al. 1997). Subsequently, several diazotrophic endophytes were isolated from different cultivated and wild rice species, and their genetic diversity was studied using polymerase chain reaction-based techniques (Barraquio et al. 1997; Stoltzfus, de Bruijn 1999; Stoltzfus et al. 1997). The bacteria isolated from rice also included certain novel diazotrophic endophytes belonging to *Serratia* and *Alcaligenes* species (Hurek et al. 1999) as well as, on some occasions, the rhizobial strains (Yanni et al. 1997). Interestingly, *Alcaligenes* species inhabiting rice were always seed-borne endophytes, whereas *Serratia* species appear to infect rice grown in the soil (Hurek et al. 1999). Endophytes usually infect rice roots through the epidermis or lateral root cracks (Barraquio et al. 1997; Cocking 1999; James et al. 1999; Reddy et al. 1997; Reinhold-Hurek, Hurek 1998; Sevilla, Kennedy 1999). Endophytes such as *Serratia* and *Azoarcus* were aggressive colonizers, predominantly establishing in xylem vessels and aerenchyma (Gyaneshwar et al. 1999; Hurek et al. 1999). The flavonone naringenin stimulated the colonization of *Azorhizobium caulinodans* and certain endophytes through lateral root cracks (Webster et al. 1997; Ladha et al., unpublished). Observations of *nif* gene expression in endophytes such as *Azoarcus* and *Herbaspirillum seropedicae* suggest that, in the rice roots, conditions are conducive to nitrogen fixation (Egener et al. 1998; James et al., personal communication; Hurek et al. 1999). Recent studies also evidenced that rice harbors a diverse array of nonculturable endophytic diazotrophs, which probably make a significant contribution to nitrogen requirement of the plants (Hurek et al. 1999). Compelling evidence also shows that some endophytes, especially some rhizobia, promote plant growth other than by supplying N through nitrogen fixation (Dazzo et al. 1999; Phillips et al. 1999; Yanni et al. 1997; Biswas et al., unpublished).

6. Determining the Genetic Predisposition of Rice for Symbiosis with Rhizobia

Legume-rhizobial interactions culminate in the formation of structures known as nodules, where rhizobia, insulated from microbial competition, fix nitrogen. This N then becomes directly available for plant growth. A long-standing goal of biological nitrogen fixation research has been to extend the nitrogen-fixing symbiosis to nonnodulated cereal plants such as rice (see Khush, Bennett 1992; Reddy, Ladha 1995). In the past, several researchers examined rice-rhizobia interactions and reported the induction of “nodule-like structures”/“hypertrophies”, albeit at extremely low frequencies (in 0.1–0.2% of the plants), upon inoculation of rhizobia either to normal roots (Bender et al. 1990; de Bruijn et al. 1995; Rolfe, Bender 1990) or to enzyme-treated roots in the presence of polyethylene glycol and calcium chloride (Al-Mallah et al. 1989). In our studies, we relied on the extensive knowledge of legume-rhizobia symbioses to formulate strategies for developing rice-rhizobia symbioses. Adopting a systematic approach, we initially addressed the following questions to evaluate the extent of predisposition of rice to form an intimate association with rhizobia:

- Do rice root exudates induce the expression of the nodulation (*nod*) genes in rhizobia?
- Do rhizobia elicit morphological and developmental responses in rice roots?
- Do rhizobia provoke a defense response in rice?
- Do rice roots perceive Nod signals and, if so, is the signal transduced appropriately?
- Does rice possess homologues of legume early nodulin genes and, if present, are their regulatory mechanisms similar to those in legumes?

Recent research has found that *Rhizobium* and rice roots interact and that many of the elements found in the legume symbioses are also present in rice. For example, for *Rhizobium* to infect the legume root, the bacterial *nod* genes must be induced by plant-produced flavonoids. Our studies indicated that the roots of specific rice cultivars exude compounds that are able to induce, albeit to a low extent, the transcription of the *nod* genes of *Rhizobium* sp. NGR234, a rhizobial species that has an extraordinarily large host range (Reddy et al. 1999b; Rolfe et al. 1999). But neither rhizobia nor purified Nod factors could elicit root hair deformation or cortical cell divisions leading to true nodule development in rice (Reddy et al. 1997).

A primary mode of rhizobial invasion of rice roots is through cracks in the epidermis and fissures created during the emergence of lateral roots (Reddy et al. 1997; 1999b). This infection process, unlike in legumes, is *nod*-gene independent, and does not involve the formation of infection thread. Moreover, rhizobial invasion provoked a mild defense response localized in the vicinity of the site of colonization. During legume nodulation, specific plant genes, known as early nodulin (*ENOD*) genes, are induced and are required for normal nodule development. Reddy et al. (1999a) showed that several legume *ENOD* genes hybridized to DNA from a variety of rice genotypes. This work was subsequently confirmed by isolating rice cDNAs showing considerable sequence homology to legume *ENOD93* (Reddy et al. 1998a) and *ENOD40* (Kouchi et al. 1999).

Another requirement for legume nodulation is the ability of the legume to respond to the rhizobial Nod signal, and the consequent signal-induced expression of the *ENOD* genes necessary for nodulation. Studies revealed that rhizobial Nod factors can induce the expression of legume *ENOD12* promoter in rice (Reddy et al. 1998b; Werthmuller et al. 1998). The results with *ENOD12*-GUS transgenic plants are intriguing because they indicate that rice possesses not only the mechanism to recognize rhizobial Nod signals but also the signal transduction chain that links such recognition to *ENOD* gene transcription. In addition, similar to the situation in legumes, the expression of the legume *ENOD40* promoter is induced only in the vascular tissues in rice (Reddy et al. 1999b). Likewise, the expression of rice *ENOD40* in soybean nodules is also restricted to the vascular bundles (Kouchi et al. 1999). These findings clearly suggest that legume and rice *ENOD40*s share a similar regulatory mechamsm(s).

In legume-*Rhizobium* symbiosis, the host plant provides the genetic information for the development of nodules. The role of the rhizobia is to trigger the host-plant genes to facilitate infection and help create a nodular-niche for nitrogen fixation (see Denarie et al. 1996; Long 1996). A monocot plant such as rice would unlikely possess the complete complement of genes or genetic programs involved in the nodule ontogeny. Rice, however, although it does not develop a symbiotic association with rhizobia, is able to enter into symbiotic associations with mycorrhizal fungi (see Khan, Belik 1995). Genetic links between the processes involved in nodulation and arbuscular mycorrhizae have been found in legumes (Albrecht et al. 1998, 1999; Gough, Denarie 1999; Denarie, Gianinazzi-Pearson 1997). For example, studies on nodulation mutants of pea have demonstrated that the early nodulin genes *ENOD2, ENOD11, ENOD12,* and *ENOD40,* which control the initial steps of nodulation, also govern the early stages of mycorrhizal development (van Rhijn et al. 1997). Thus, because rice is able to form symbiotic associations with mycorrhizal fungi, and because the formation of such an association of mycorrhizal fungi with legumes is mediated by *ENOD* genes, we can infer that at least some of the genetic mechanisms required to promote endosymbiosis with rhizobia likely exist and function in rice. The challenge then becomes to identify the plant traits that are unique to rhizobial nodulation so that they can be engineered into the rice plant.

In conclusion, certain critical differences exist between the association of rice and rhizobia relative to the root-nodule symbiosis with legumes. Some of the molecular interactions that occur in these plant-microbe associations, however, may be similar. It is therefore essential that studies be extended at the cellular and molecular levels to identify why such responses do not fully occur in rice in order to contemplate genetically engineering this major cereal crop to form a more intimate endosymbiotic association with rhizobia.

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GENETIC TRANSFORMATION OF LEGUMES

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1. Introduction

As a group, legumes have been considered difficult to transform. While some species have remained recalcitrant many have now been successfully engineered to express a variety of novel traits. The list of transformed legumes is almost entirely restricted to genera of the Papilionoideae and, not too surprisingly, to species that are used for food and forage. They include 22 species of pulse and 14 pasture species (Atkins, Smith 1997). Among woody legumes transformation has been reported for *Robinia pseudoacacia* (Han et al. 1993) and a species of *Acacia* (Quoirin et al. 1997). The frequencies of transformation are considerably lower than those for solanaceous species and are comparable to those with cereals (<1-5%). There are significant species and cultivar variation (Hamblin et al. 1998). For example varieties of *Lens culinaris* transform with frequencies of 1-5% while *Cicer arietinum* shows frequencies of 0.1-0.3% and, among a range of cultivars of the narrow leafed lupin (*Lupinus angustifolius*), transformation frequencies vary from as low as 0.01% to as high as 3%. *Lupinus luteus* shows frequencies of 0.5% or lower while *Lupinus albus* has proven impossible to transform. *Vicia faba* has also been recalcitrant while *V.narbonensis* has been transformed successfully (Saalbach et al. 1995). In soybean also, significant cultivar specificity has been shown and, although a wide range of transformation methods have been applied, the frequency of viable, transgenic seed recovered is usually low (Trick et al. 1997). Despite this limitation, transgenic soybean cultivars expressing herbicide tolerance (Padgette et al. 1995) and resistance to Lepidopteran insects (Stewart et al. 1996) have been commercialised and are now well established in the agricultural production systems of some countries.

2. Regeneration of Transformants

While there are many factors that together contribute to the low recovery of transformants a major problem has been in regenerating an intact viable plant. As a result, numerous highly species-specific tissue culture techniques and media have been devised which in many cases rely on ‘highly regenerable’ cultivars (e.g. the line in *cv.* Jemalong 2HA of *Medicago truncatula* used by Wang et al. 1996). However, crop improvement through genetic engineering must use commercially viable elite cultivars or breeding lines to maximise the benefit of inserting transgenes. Thus, more generic protocols that are less or not dependant on particular genotypes are necessary.

The use of developing, mature or germinating embryonic axes goes some way towards satisfying this need by removing the complexity and culturing problem of transforming cells and regenerating through callus induction and subsequent organ differentiation. In some cases germinating embryo tissue permits adventitious regeneration while in others regeneration proceeds through axillary shoot growth. The use of germinating embryo explants with essentially the same *Agrobacterium*-mediated protocol has proven successful with narrow-leafed lupin, yellow lupin, pea, chickpea, lentil and *Trifolium pratense* (Hamblin et al. 1998). Similar techniques using *Agrobacterium* to transform cotyledonary node explants in short season soybean generated a transformation rate of 27-92% but the transformation events were mostly restricted to non-regenerable callus and only a single viable transgenic plant was recovered from almost 200 selections (P.A. Donaldson and D.H.

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Simmonds, unpublished). Embryogenic suspension cultures derived from developing soybean cotyledons have also proved amenable to either biolistic or *Agrobacterium*-mediated transformation with sonication greatly enhancing the frequency (Trick, Finer 1998). While these methods return a higher frequency of transgenics than *Agrobacterium* infection of cotyledonary nodes significant genotype specificity has also been noted (D.H. Simmonds and P.A. Donaldson, unpublished). Adjustment of the relative proportions of growth regulators (auxin and cytokinin) in culture media induces rooting but this varies widely among species and in some cases imposes a further recalcitrant step. Where root growth has proven to be extremely difficult to induce transformed shoots are grafted onto untransformed rootstocks (Pigeaire et al. 1997).

More recently a number of *in planta* methods have been developed using direct insertion of DNA with electroporation (Chowira et al. 1995; 1996) or *Agrobacterium*-mediated transfer (Oger et al. 1996). These techniques eliminate the need for tissue culture and *in vitro* regeneration; relying on the intact plant to support the growth of transformed meristems that yield transgenic seed on an otherwise untransformed parent. Initial reports on *in planta* electroporation indicated a very high frequency of transformation with GUS (Chowira et al. 1996) in a wide range of species, but the method appears not to have been more widely adopted and as yet the nature and stability of transformants has not been reported.

3. Transformation Methods

Recent reviews (Atkins, Smith 1997; Christou 1996) identify transformation based on the use of *Agrobacterium tumefaciens* carrying a binary vector as the most commonly used method and probably the most successful overall. *A. rhizogenes* transformation by induction of 'hairy roots' has found favor with a number of pasture legumes that propagate readily through root or stolon segments (Webb et al. 1990). Among the direct methods, bombarding the tissue or cells with microprojectiles coated in DNA (the "Particle Gun" method; Christou 1992) has been applied widely. In the case of soybean a biolistic approach has also been successful with embryogenic suspension cultures generated from immature cotyledon tissues in the presence of high levels of 2,4-D (Hadi et al. 1996; Trick et al. 1997). More recently co-culture of *Agrobacterium* with embryogenic cultures following sonication (Sonication-Assisted-Agrobacterium-mediated-Transformation, SAAT; Trick, Finer 1998) has yielded encouraging results. There has been renewed interest in electroporation with the demonstration that it can be applied *in planta* (Chowira et al. 1995; 1996). A variation on the *in planta* idea is the direct infection of intact shoot meristems of *Lotus japonicus* with *A.tumefaciens* (Oger et al. 1996).

Vacuum infiltration to assist *Agrobacterium* infection of intact tissues has been successful with *Arabidopsis thaliana* (Bechtold et al. 1993) and a recent report indicates that this technique has been applied to *Medicago truncatula* (M. Harrison reported in Cook 1999). The most recent method used with *Arabidopsis* has eliminated the need for vacuum infiltration (Clough, Bent 1998) and simply involves dipping floral tissues into a suspension of *Agrobacterium* containing sucrose and a surfactant. The transformation frequencies with these methods is still modest (0.5-3%; Clough, Bent 1998) but their simplicity and the complete elimination of tissue culture allows very high throughput.

The strain of disarmed *A.tumefaciens* used has been shown to have a significant impact on transformation frequency (Pigeaire et al. 1997). In lupin, one (AgLO, Lazo et al. 1991) of four different strains each carrying the same binary vector (pCGP83, Janssen, Gardner 1989) containing a *gus* construct proved to be the most effective. Testing a range of *Agrobacterium* strains with species whose transformation frequencies are at present very low may lead to improvement. In a number of cases acetosyringone application has been used to improve *Agrobacterium* infection (e.g. Trick, Finer 1998) but systematic study of a range of possible ‘enhancers’ is yet to be undertaken.

4. Selectable Marker Genes

Although initially a number of genes that confer resistance to antibiotics were used these have been largely discarded in favor of genes that confer resistance to herbicides. Most commonly used is the *bar* or *pat* gene (Thompson et al. 1987; Strauch et al. 1988). These two genes were cloned from different *Streptomyces* and encode the enzyme phosphinothricin acetyltransferase that detoxifies the active ingredient (phosphinothricin or glufosinate) of the herbicide Basta®. Basta® is extremely effective and explants in culture are rapidly killed in the absence of either of the resistance genes. Both plant and bacterial genes that confer resistance to glyphosate or Roundup® have also been used (Zhou et al. 1995; Fuchs et al. 1993). Resistance genes for a number of other herbicides have also been identified and these will extend the range of selectable markers for use in legume transformation (Chandler 1995). While the *bar* gene has proven to be an extremely useful tool in selecting transformants its widespread use as a marker poses some serious problems.

Incorporation of resistance to Basta® in a range of crops that might share a farming system is not desirable from a management point of view and increases the likelihood of transferring the *bar* gene to wild and weedy relatives. Similarly, there are legitimate community concerns over the widespread use of both herbicide and antibiotic resistance genes in food crops (Zechendorf 1994). A number of possible means to excise a selectable markers have been proposed (Dale, Ow 1991; Yoder, Goldsborough 1994; Komari et al. 1996) as a possible solution to this problem. Recently Gleave et al (1999) have recovered marker-free tobacco plants without sexual crossing using transient expression of the *cre* recombinase to mediate excision of genes flanked by *lox*P. Progress in understanding gene silencing (Kooter et al. 1999), largely as a result of the initially unpredictable and variable expression of transgenes, might hold the key to developing genetic tools for preventing the expression of selectable markers. For these reasons alternative selectable markers based on genes that confer benign phenotypes, for example genes that allow nutritional selection analogous to microbial auxotrophy or that exploit the toxicity of antimetabolites or heavy metals, are being sought.

5. Transformation Strategies to Enhance the Value of Nitrogen Fixation by Legumes

Apart from their enormous commercial value as a source of protein, oil and forage, legumes, through their capacity to reduce dinitrogen, potentially contribute to the development of more sustainable agricultural production systems. In this regard it is relevant to ask- can the value of legumes be enhanced either by increasing the potential for N_2 fixation or by improving the efficiency of its operation? A related secondary question is whether transgenic technology can be exploited to achieve such enhancement.

Clearly the speed with which effective nodules form on seedlings will reduce the period of ‘N-hunger’ exhibited by many legumes in the field (Fred et al. 1937). Even short periods of transient N-deficiency in early vegetative growth may not be completely overcome and can lead to significant depression of grain N and harvested yield (Ma et al. 1998). Rapid progress in understanding the molecular events in processes of recognition, infection and early nodule development will provide a basis in the near future for altering plant gene expression to enhance symbiotic establishment. No doubt continued selection of *Rhizobium* strains and improved inoculation methods together with a clearer appreciation of bacterial ecology in the rhizosphere will also contribute.

However, strategies to enhance the rate of N_2 fixation and its value to the plant are not so obvious. Earlier studies of the C/N economy of nodules on different legumes established that there are significant differences in the amount of translocated C required for fixation and the export of fixed N (Layzell et al. 1979). The differences appear to be due to the proportion of C allocated to respiration, conservation of respired CO_2 and synthesis of the translocated N-solutes (asparagine versus ureides) as well as small differences due to net H_2 evolution. Application of a transgenic approach to establish the importance of these traits and identify critical genes that might be used to alter their expression and regulation is lacking.

Nodules are phloem fed organs that establish their 'sink-strength' within the competitive C-allocation patterns of the plant. Their capacity for N_2 fixation is thus a direct function of their sugar supply. These patterns of allocation change throughout growth and at the onset of reproductive development and during seed filling nodule activity declines markedly. Although enhancing the persistence of a functional symbiosis may be a desirable goal the mechanisms that regulate phloem transport to and of unloading assimilates within nodules are not known. It seems likely that not only assimilates and other small molecular weight solutes but also information molecules in the form of growth regulators including bioactive peptides (Narvaez- Vasquej et al. 1995), proteins and oligonucleotides (mRNA) are translocated in phloem (Lucas, Wolf 1999; Thompson, Schultz 1999). Indeed the long distance translocation of low levels of mRNA in sieve tubes has been interpreted as a mechanism for gene-specific systemic acquired silencing in sink tissues (Jorgensen et al. 1998). Recognition of the importance of these factors in establishing the nature and patterns of translocation to other phloem-fed sinks, such as vegetative apices, seeds and tubers, is beginning to emerge, and a transgenic approach to altering the biochemistry of sugar utilization and synthesis of stored reserves in these organs has been initiated (Herbers, Sonnewald 1998; Roitsch 1999). A similar application of these tools to developing legume nodules is a clear research imperative that may lead to new genetic strategies for enhancing their competitive sink-strength. Although manipulation of invertase or the expression of other single enzymes of sugar metabolism has not led to increased partitioning of C to harvestable sinks (Herbers, Sonnewald 1998), these transgenics have revealed something of the signal transduction pathways involved in regulating the allocation of resources.

There are negative impacts of legume culture on the sustainability of soil resources- more specifically the accelerated acidification of upper soil horizons. N_2 fixation is regarded as less prone to cause localized acidification compared to the assimilation of NO_3 (Raven, Smith 1976) and so enhancing the contribution made by the symbiosis is likely to be beneficial. However, there is very substantial genetic diversity among pulse species in the extent to which H^+ are excreted (Ritchie et al. 1993). Whether or not there is also diversity within a species remains to be established. In any case, the mechanisms that led to these differences in expression and their encoding genes are not known.

6. Model Legume Species

There is little doubt that exploiting the genetic features of *Arabidopsis* as a model has revolutionised basic studies of plant growth and development and it is reasonable to suppose that exploiting a model like *Medicago truncatula* (Cook 1999) or *Lotus japonicus* (de Bruijn et al. 1997) will similarly benefit our understanding of legume symbioses. The nodulated legume also offers a basis for the study of other plant-microbe interactions and it is likely that a genetic analysis of these model systems will benefit this broader area as well (Ausubel, Bisseling 1999). Regeneration of transgenics in both of these species has suffered from the same low frequencies typical for most legumes. However, recent development (M. Harrison, as cited by Cook [1999]) of a method for vacuum infiltration with *Agrobacteruim* along lines similar to *Arabidopsis* offers the promise of frequencies high enough for generating T-DNA tagged mutant libraries. Such a development will have a significant impact on this area of research.

No doubt these models will reveal considerable synteny between legume species allowing cross-referencing of mapping data for many symbiotic traits. However, nodule initiation and development in *Medicago* is only typical of some symbioses while that of *Lotus* is typical of only some others. Those that develop nodules without a persistent meristem and produce ureides rather than asparagine as exported products of fixation (Atkins 1991), or those, in which the infection process does not rely on root hair invasion, may not be so well served. Pasture legumes, like *M.truncatula* or *L.japonicus* have a relatively indeterminate flowering habit and very different source-sink relations compared to the more determinant high protein-grain pulse crops. Thus seeking genes and promoters for traits like enhanced efficiency of sugar usage or for the establishment and persistence of 'sink strength' of nodules in the model species currently in vogue may not be fruitful.

7. Conclusions

Routine transformation technology for most important pulse and pasture legume species has been developed and, in some cases, successfully applied. Early methodology suffered from low transformation frequencies largely as a result of poor regeneration techniques, poor reproducibility and limited genotype specificity. These limitations are being overcome. Current selectable markers based on herbicide or antibiotic resistance are highly effective research tools but are no longer acceptable for practical genetic engineering and the release of a new generation of transgenic cultivars. It is necessary that markers with more benign phenotypes become available. Transgenic strategies to enhance the value of legumes through altering symbiotic features are yet to be developed. Current knowledge of the early events in nodule establishment should allow their application in this area relatively soon. However, the efficiency with which phloem-supplied C is utilized in nodules to export fixed N and the ability of nodules to maintain their 'sink strength' and attract assimilates within the competitive allocation of C-resources in the plant hold the keys to enhancement. Our knowledge of the mechanisms that regulate phloem allocation of assimilates and the establishment of source- sink relation is inadequate. So much so, that transformation methods that have been developed are a technology awaiting the genes and promoters so it can be applied.

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CAN THE STUDY OF ENDOMYCORRHIZAE OPEN NEW AVENUES OF RESEARCH IN SYMBIOTIC NITROGEN FIXATION?

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1. Introduction

The study of the interaction of plants with diazotrophic bacteria has clearly shown that only endophytic associations provide significant amounts of fixed nitrogen to the host plant. The *Rhizobium*-legume symbiosis, which is one of the most efficient biological nitrogen fixation systems known (see Mylona et al. 1995), is a good example. At least as much ammonia is produced annually by this symbiosis as by the whole chemical fertiliser industry world-wide. The fact that nitrogen fixation by the *Rhizobiutn*-legume symbiosis provides such significant benefits to host plants is due to a number of features, including the formation of specific organs, the nodules, and the intracellular nature of bacteria, which protects the microsymbiont from the competition with other rhizosphere microbes and allows efficient metabolic and signal exchange at the cellular level among the partners. The ability to form efficient nodules is under the control of large numbers of plant genes (see Mylona et al. 1995) and the challenge of transferring this ability to non-legumes such as cereals represents a very long term vision. In contrast, the possibility of developing cereals capable of other forms of endosymbiotic nitrogen-fixation seems to be a more modest and realistic challenge. Here, we discuss the possibilities that endomycorrhizae might open this avenue.

2. Arbuscular Mycorrhizae, an Ancient and Widespread Endosymbiosis

While relatively few plants are able to form nodules in association with nitrogen-fixing bacteria, the ability of plants to form root endosymbiotic associations with micro-organisms is much less restricted. In fact, the majority of plants, including crops such as wheat, maize and rice, are able to establish root endocellular symbioses with arbuscular mycorrhizal fungi (Smith, Read 1997, Gianinazzi-Pearson, 1996; Harrison 1999). Arbuscular mycorrhizae (AM), formed between the roots of most higher plants and obligate biotrophic fungi of the order *Glomales,* are by far the most common root endosymbiotic association. The AM symbiosis is extremely ancient and structures identified as arbuscules have been found in fossil tissues of an early Devonian land plant, indicating at least a 400 million-year-old association between terrestrial plants and AM fungi (Remy et al. 1994). AM fungi procure and transport phosphate and other nutrients from the soil to plant roots. In turn, the host plant provides fixed carbon to its fungal partner (see Harrison 1999). In contrast to the *Rhizobium*-legume association, there is very little host specificity in AM symbioses. Fungal hyphae contact roots via specific structures, called appressoria, from which hyphae entering the root are produced. Hyphae grow either inter- or intracellularly through the epidermis and the outer cortex, depending on the host plant. In the case of intercellular infection, the process which has been most commonly studied, hyphae enter the root between two epidermal cells. In the inner cortex, the fungus invades root cells, and arbuscules are generated. These are highly ramified structures thought to play an important role in metabolic exchange between the two partners. Although the fungus is intracellular at this stage, it is not in direct contact with the host cytoplasm, since a perifungal membrane, originating from the plant plasma membrane, invaginates and surrounds the arbuscules.

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The plant genetic control of endomycorrhization has not yet been studied in detail, mainly because of the difficulty in screening for mutants, but also because of the recalcitrance of the fungal partner to growth in pure culture. Moreover, the model plant *Arabidopsis thaliana,* cannot form arbuscular mycorrhizae, precluding its use as a genetic system to study plant-mycorrhizal interactions.

3. Common Steps in Nodule and Endomycorrhizae Formation in Legumes

Many parallels exist between root nodule formation induced by rhizobia and root colonisation by AM fungi (Albrecht et al. 1999; Harrison 1999). In both cases during the penetration of root tissues, the microsymbionts are separated from host cells by plant cell wall-derived material and the plant plasma membrane. In both cases the target tissue for the microsymbiont is the root cortex, and extensive defence reactions are not triggered. The intracellular invasion and colonisation of cortical cells is linked to reactivation of the host cytoplasmic compartment and nucleus as well as proliferation of the microsymbiont and its development into specific structures, the bacteroids in nodules and the arbuscules in mycorrhizae. Both bacteroids and arbuscules are separated from the plant cytoplasm by a host derived perisymbiotic membrane. The peribacteroid membrane (in nodules) and the periarbuscular membrane (in mycorrhizae) are extended surfaces between the partners that facilitate bi-directional exchange of metabolites. It has been found that certain infection thread and peribacteroid compartment glycoconjugates (MACs) are immunodetected in plant-derived components of the arbuscule interface (Perotto et al. 1994; Gianinazzi-Pearson 1996).

Most legumes are able to establish root endosymbioses with both AM fungi, to form arbuscular mycorrhizae, and with rhizobia to form nodules. Pioneering genetic studies have led to the finding that certain nodulation (Nod$^-$) mutants of pea and of *Vicia faba* are simultaneously altered in their ability to form arbuscular mycorrhizae (Myc$^-$), while their ability to interact with soil pathogens is unaffected (Duc et al. 1989; Gollotte et al. 1993). This shows that some "common" symbiotic genes exist in legumes, that are essential for both the rhizobial and the endomycorrhizal symbioses. Four such loci have now been identified in pea (Albrecht et al 1999) and Nod$^-$Myc$^-$ mutants have been characterised in other legume species, including *Medicago truncatula* (Sagan et al 1995, 1998), bean (*Phaseolus vulgaris*) (Shirtliffe and Vessey 1996) and *Lotus japonicus* (Szczyglowski et al 1998; Wegel et al 1998). Interestingly, the *sym8* gene of pea, which is one of the four genes controlling both nodule and endomycorrhizae formation, has been found to control a step of the signal transduction cascade, induced by the rhizobial nodulation signals, the Nod factors, as well as by AM fungi, and leading to expression of the early nodulin genes *ENOD5* and *ENOD12* (Albrecht et al 1998). Other early nodulin genes have been identified that are also induced during AM formation – *MsENOD2* and *MsENOD40* in *Medicago sativa* (Van Rhijn et al 1997), and a leghemoglobin gene (*VfLb29*) in *Vicia faba* (Frühling et al. 1997).

4. Most Crops Possess a Genetic Program for Root Endosymbiosis

In the *Rhizobium*-legume symbiosis, cross inoculation and genetic experiments show that it is the plant partner that possesses the genetic program that specifies the type and pattern of infection and the ontogeny, anatomy and histology of nodules (Dénarié et al. 1992). One role of the microsymbiont is to trigger the infection and developmental program of the host by specific symbiotic signals (Dénarié et al 1996). Similarly, cross-inoculation experiments reveal that it is the plant partner which determines the site of entry, the pattern of infection and internal development of arbuscular endomycorrhizae (Harrison 1999). Genetic evidence confirms the existence of plant genetic programs for AM formation. Certain phylogenetic groups of plants, for example the majority of the members of the Brassicaceae (including *Arabidopsis thaliana*) and Chenopodiaceae, cannot form endomycorrhizae. In addition, plant mutants have been identified, in legumes and tomato, that block AM formation at different stages: colonisation at the root surface (Barker et al. 1998), appressorium formation (Duc et al. 1989, Sagan et al. 1995,), abortion of hyphal penetration in the epidermis (Shirtliffe, Vessey 1996; Wegel et al. 1998), and absence of differentiation into arbuscules (Gianinazzi-Pearson 1996). This indicates that most plants possess a complex genetic program controlling various aspects of AM development. Moreover, since both legumes and non-legumes are able to establish arbuscular mycorrhizae with the same fungal species, it is reasonable to assume that "common" symbiotic genes homologous to those identified in legumes are also involved in controlling AM formation in non-legumes,

5. *Medicago truncatula* Genes Controlling Nodule and Endomycorrhizae Formation

Arabidopsis thaliana is unable to establish endosymbiotic associations. In contrast, legumes are able to establish both types of symbioses and are thus attractive systems to identify and characterize symbiotic genes. Many legumes have however large or complex genomes which render their molecular genetics difficult, and so *Medicago truncatula* was proposed as a model legume (Barker et al 1990) and a number of laboratories world-wide have now decided to concentrate their research efforts on this species (Penmetsa, Cook 1996; Charon et al. 1997; Cook et al. 1997; Liu et al. 1998; Cook et al., this book). *M. truncatula* was chosen because of its amenability to molecular genetic analysis (diploid, genome size of ~500 Mb, efficient transformation with *Agrobacterium*) and because its microsymbiont, *Sinorhizobium meliloti,* is the most extensively studied rhizobial species. Co-ordinated projects are underway to sequence the chromosome and two symbiotic megaplasmids of *S. meliloti* (Kahn et al., this book). Two extensive mutageneses have been performed on *M. truncatula* by gamma rays (Sagan et al 1995, 1998) and ethyl methane sulfonate (Penmetsa and Cook 1996) and diverse types of mutants altered in nodulation and its regulation have been identified. The analysis of their mycorrhizal phenotype has identified both Nod$^-$Myc$^+$ and Nod$^-$Myc$^-$ mutants. Characterization of Nod$^-$Myc$^+$ mutants has so far led to the identification of a gene involved in the Nod factor transduction cascade and of a gene specifically involved in the control of infection initiation (R. Catoira, T. Timmers, C. Gough, C. Galera, F. Maillet, V. Penmetsa, D. Cook, C. Rosenberg, G. Truchet and J. Dénarié, manuscript in preparation).

Analysis of Nod$^-$Myc$^-$ mutants revealed the existence of at least two complementation groups (Sagan et al. 1995, 1998; M. Harrison, V. Gianinazzi-Pearson and D. Morandi, personal communications). Further genetic analysis, in cooperation with E. P. Journet, has enabled the identification of three genes (*Mtdmi1, Mtdmi2* and *Mtdmi3*) involved in early stages common to both symbioses (R. Catoira, C. Galera, C. Gough, F. de Billy, F. Maillet, V. Penmetsa, D. Cook, C. Rosenberg and J. Dénarié, manuscripts in preparation). The *M. truncatula* Nod$^-$Myc$^-$ mutants studied so far are pleiotropically affected in their responses to Nod factors; they are affected for early nodulin gene expression and for root hair deformation, while retaining their ability to specifically recognise Nod factors, strongly suggesting that *Mtdmi1, Mtdmi2* and *Mtdmi3* encode components of the Nod factor signal transduction cascade. We hypothesize that these three genes control steps common to the transduction of both nodulation (Nod factors) and mycorrhization symbiotic signals. By analogy to the *Rhizobium* symbiosis we hypothesize that AM fungi trigger the plant mycorrhizae program by specific symbiotic signals, "Myc factors". Groups in Toulouse are currently trying to purify and characterize these putative signal molecules, by an interdisciplinary approach. Once the chemical structure of Myc factors is deciphered, it might be possible to engineer nitrogen-fixing bacteria, rhizobia for example, to synthesize these signals and then study their behaviour and plant responses to such Myc factor-producing bacteria.

Nod$^+$Myc$^-$ mutants, being more difficult to screen for, have not yet been identified. But the isolation and genetic analysis of such mutants with a range of mycorrhiza-defective phenotypes would lead to the identification of genes specifically involved in the establishment and functioning of mycorrhizae and hence a better understanding of the genetic complexity of AM symbiosis.

6. *M. truncatula* Genomics to Systematically Identify Symbiotic Genes

To have a more complete picture of the genetic programs for root endosymbioses, in addition to identifying genes by studying mutants, it is important to study whole sets of genes expressed at different times during symbiotic interactions. Molecular approaches, which are useful complements to genetic analysis, have been successfully used in *M. truncatula* to identify plant genes whose expression is modified during nodulation (Gamas et al. 1996, de Carvalho Niebel et al. 1998) or mycorrhization (Burleigh and Harrison 1997, van Buuren et al. 1999). Structural and functional genomics constitute novel approaches to identify large numbers of symbiotic genes and considerable progress is being made in these fields for *M. truncatula* (Cook et al., 1999 and this book). Firstly, tools are being developed for map-based cloning of *M. truncatula* symbiotic genes. These include a genetic map (T. Huguet, Toulouse), a BAC (Bacterial Artificial Chromosome) genome library (D. Cook, Texas A&M University) and physical mapping by FISH (Fluorescent In Situ Hybridisation) technology (T. Bisseling, Wageningen). A number of genes involved in Nod factor recognition, or controlling nodulation and nodulation/mycorrhization are currently in the process of being cloned. For example, *Mtdmi1* and *Mtdmi2,* as well as *Mtnfr1,* a gene involved in Nod factor recognition, have been precisely located on the genetic map of *M. truncatula* (T. Huguet, personal communication) and are now being isolated, by map-based cloning, in collaboration with T. Huguet (INRA-CNRS, Toulouse) and D. Cook (Texas A&M University).
Secondly, a large scale T-DNA activation tagging project is underway (M. Harrison, Noble Foundation, USA), and aims to generate 300,000 tagged lines which will be screened for symbiotic mutants. Thirdly, in order to systematically identify and characterise genes expressed specifically during each symbiosis and genes expressed in both symbioses, EST (Expressed Sequence Tag) libraries are being generated in the USA (NSF Plant Genome Program, Covitz et al. 1998, Cook et al. 1999) and in Europe (see Gamas et al., this book). These programs should allow the identification of a very large proportion of the legume genetic program for root endosymbioses.

7. Rice Functional Genomics to Study the Genetic Program for Root Endosymbiosis of Cereals

The global study of gene function by functional genomics is underway in rice, which has been chosen as a model monocotyledonous plant (Izawa and Shimamoto 1996; Bennetzen 1999), because of its small (~440Mb) diploid genome and its ability to be easily transformed. Comparative molecular genetic mapping has indicated a similar gene content and extensive map colinearity between rice and other grasses, suggesting that much information acquired from rice can be applied to other major food crops, such as wheat, maize and barley. In addition, both upland and wetland rice varieties are able to form endosymbiotic associations with AM fungi (Khan et al. 1995).

A large proportion of rice genomics is being achieved by the Rice Genome Research Program in Japan (RGRP, National Institute of Agrobiological Resources, Tsukuba, Japan). Since 1991, the RGRP has carried out large-scale EST sequencing, construction of a fine-scale restriction fragment length polymorphism (RFLP) map and physical mapping of the rice genome with yeast artificial chromosome (YAC) clones (Yamamoto and Sasaki 1997). The sequencing of the complete genome of Nipponbare (a japonica variety of rice), started in 1998 (Sasaki 1998). Although cDNA sequencing has been performed on a very large scale in rice, and has catalogued organ-specific, developmental-stage specific and stress-specific genes, to our knowledge no EST libraries have been made from mycorrhized rice roots. We therefore propose the generation and sequencing of such mycorrhizae-specific ESTs, the study of which would be necessary in order to identify rice genes whose regulation is modified during AM symbiosis, in other words the rice genetic program for root endosymbiosis. Within a few years, it should be possible to compare the set of rice genes involved in mycorrhization with those of *M. truncatula* involved in mycorrhization and nodulation. This should provide an experimental basis for assessment of the number of genes needed to be transferred to rice to enable, in the long term, this crop to form nodules in the presence of rhizobia.

8. The Potential Nitrogen-fixing Ability of AM Fungi

The demonstration, by genetic analysis of Nod^- mutants, that there are genes common to nodulation and endomycorrhization has important evolutionary implications. The characterization of Myc factors, as well as the analysis of plant responses to these factors, would constitute crucial steps towards a better understanding of the genetic basis of root endomycorrhization. Unfortunately, though, this knowledge probably will not enable us to suggest short-medium term strategies to make cereals able to establish endosymbiotic associations with nitrogen-fixing bacteria. However, the presence of bacterium-like organisms (BLOs) detected in the cytoplasm of many AM fungi may change this scenario. In the strain *Gigaspora margarita* BEG 34, where BLOs have been studied in detail by electron microscopy and PCR approaches (Bianciotto et al., 1996), they can be present in very large numbers in spores (approx. 250,000 per spore). They appear to be obligate endosymbionts and are present in both spores and hyphae in successive generations (V. Bianciotto, personal communication). Molecular phylogenetic analysis showed that they belong to the genus *Burkholderia* (Bianciotto et al. 1996, Perotto, Bonfante 1997). The presence of DNA sequences homologous to *nifDK* genes of *Azospirillum brasilense* has recently been reported in the BLOs of *Gigaspora margarita* (Minerdi et al. 1998; D. Minerdi, R. Fani, R. Gallo and P. Bonfante, submitted for publication). This finding that AM fungi's BLOs contain putative *nif* genes, suggests that they may have the capacity to fix nitrogen. It remains to be determined whether or not this strain of *G. margarita* has the ability to fix nitrogen, if nitrogen fixation can occur during symbiosis within roots of host plants and, if so, whether fixed nitrogen is transferred to the plant.

9. Perspectives

In the near future, genetic and genomic approaches will provide a wealth of information on the plant genetic programs for root endosymbioses, that is the cloning of symbiotic genes and the identification of hundreds of genes whose expression is modified in the course of nodulation in legumes and of mycorrhization in both legumes and cereals. In the short-medium term, this information should be useful in breeding legumes to exploit symbiotic nitrogen fixation and mycorrhization better, and in breeding rice and other important cereals for better mycorrhization. Comparison of the genetic programs for root endosymbioses in legumes and rice should allow the definition of sets of genes involved in endomycorrhization, in nodulation and in both types of symbioses. This information is required for a critical assessment of the possibility to exploit and improve the existing symbiotic genetic program of rice in such a way as to make endosymbiotic associations with nitrogen-fixing microorganisms a realistic goal, for this major crop.

Whatever the future results concerning the diazotrophy of the BLO-containing *Gigaspora margarita* BEG 34 strain, we believe the discovery that some AM fungi contain BLOs with genes homologous to *nif* genes, opens novel and extremely important avenues for research. Large scale screening of AM fungi isolated from diverse types of soils and plants, namely in soils with very low nitrogen status, might identify strains able to fix significant amounts of nitrogen. On the plant side, it is likely that modern breeding in soils enriched in phosphate (and nitrogen) fertilizers has not favoured the selection of cereal genotypes efficient for mycorrhizae formation. The cDNAs of cereal genes involved in mycorrhization, obtained in the frame of the rice genome project, might serve as molecular markers to breed for plant cultivars capable of high levels of mycorrhization, phosphate uptake and, hopefully, nitrogen fixation.

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